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Oligonucleotide Gene Therapy

PRINCIPAL INVESTIGATOR: Meredith A. McCracken

CONTRACTING ORGANIZATION: West Virginia University Research Corporation  
Morgantown, West Virginia 26506

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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b>  Protein kinase C (PKC) is a family of serine/threonine kinases composed of 12 unique isoforms that function in a variety of cellular processes including proliferation, apoptosis, differentiation and carcinogenesis. The purpose of this study was to identify specific PKC isoforms that promote breast tumor cell survival following radiation treatment. This work demonstrated that depletion of PKC $\delta$ and PKC $\zeta$ isoforms from MCF-7 and MDA-MB-231 human breast tumor cells by antisense oligonucleotides significantly impaired survival following radiation insult. Antisense oligonucleotides were shown to be effective and selective inhibitors of specific PKC isoforms. The PKC $\delta$ inhibitor, rottlerin reduced cell survival both in the presence and absence of radiation insult. Furthermore, transformation of MCF-7 cells with a PKC $\delta$ dominant negative significantly inhibited cell survival thus confirming that PKC $\delta$ inhibition attenuates breast tumor cell survival. DNA damage, assessed using the comet assay provided evidence that depletion of PKC $\delta$ from MDA-MB-231 cells by antisense oligonucleotide resulted in loss of DNA integrity while PKC $\zeta$ depletion did not. These studies have identified two PKC isoforms that function in breast tumor cell survival and support the application of isoform specific PKC inhibitors to the treatment of human breast tumors.				
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## INTRODUCTION:

Protein kinase C (PKC) participates in a variety of cellular processes. As a regulator of signal transduction pathways which control cell proliferation and death and as the primary intracellular receptor for tumor promoting phorbol esters, PKC represents a potentially important molecular target for anti-cancer therapeutics. PKC is a family of serine/threonine kinases composed of twelve unique isoforms. Relatively little information exists pertaining to the functions of individual PKC isoforms in breast cancer. However, PKC has previously been shown to have increased activity and expression in breast cancer versus normal mammary tissue (O'Brian et al., 1989; Gorge et al., 1996). The focus of the present study was on identifying specific PKC isoforms that facilitate breast tumor cell survival. Gamma radiation was used to challenge survival of the breast tumor cells in these studies because the results of earlier experiments with the PKC inhibitors, staurosporine, sangivamycin and H7 provided evidence that PKC inhibition increased radiation sensitivity (Begemann et al., 1998; Hallahan et al., 1992; Zhang et al., 1993). Antisense oligonucleotide technology was utilized to achieve isoform selective PKC inhibition. Alternative approaches to isoform selective inhibition included small molecule enzyme activity inhibitors and plasmid vectors that encode dominant negative mutant forms of PKC. Antisense oligonucleotide studies showed that specific inhibition of PKC  $\delta$  and PKC  $\zeta$  suppressed the survival of two radiation treated breast tumor cell lines. Subsequent experiments provided evidence that in the absence of radiation treatment, PKC  $\delta$  and PKC  $\zeta$  antisense oligonucleotides reduced cell survival as well. Furthermore, in the absence of radiation insult PKC  $\delta$  inhibition by both the small molecule inhibitor, rottlerin and dominant negative PKC  $\delta$  significantly reduced breast tumor cell survival. These results demonstrated that PKC  $\delta$  and PKC  $\zeta$  isoform antagonists are inhibitors of breast tumor cell survival independent of radiation exposure.

## BODY:

The first task in this project was to optimize the delivery of antisense oligonucleotides to human breast tumor cells. As reported in the July, 2000 annual progress report, various lipid delivery systems were tested for their ability to deliver a green fluorescent protein (GFP) encoding plasmid to MCF-7 breast tumor cells. However, based on inherent differences between plasmids and antisense oligonucleotides, such as size and subcellular destinations, the conditions obtained for optimal plasmid delivery could not be applied to antisense oligonucleotide delivery. Task 2 proposed to use flow sorting on the basis of GFP expression to enrich for cells co-transfected with GFP plasmid and PKC antisense oligonucleotides. This task was abandoned during the first funded year for reasons mentioned above. Through collaboration with Isis Pharmaceuticals (Carlsbad, CA), a protocol utilizing Lipofectin (Invitrogen, Carlsbad, CA) transfection reagent to deliver antisense oligonucleotides was adopted. Western blot analysis was used to confirm that Lipofectin mediated delivery of PKC antisense oligonucleotides resulted in depletion of specific PKC isoforms. Task 1- complete; Task 2- abandoned.

Task 3 examined the effect of PKC antisense oligonucleotides on PKC isoform protein expression. Protein extracts prepared from MCF-7 and MDA-MB-231 human breast tumor cells 24-96 hours after treatment with lipofectin and PKC antisense oligonucleotides were subjected to western blot analysis. Detailed descriptions of these results were presented in previous two annual reports. Briefly, PKC antisense oligonucleotides were shown to be specific and effective inhibitors of targeted PKC isoforms. Detailed descriptions are also presented in the appended manuscript (Fig. 3, McCracken et al.). Task 3- complete.

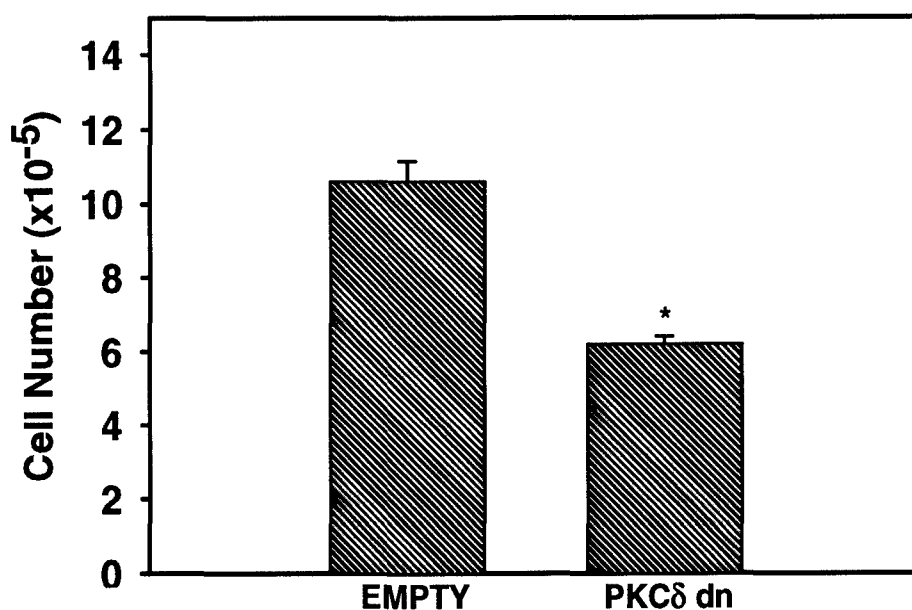
In Task 4, PKC antisense oligonucleotides targeted to five different PKC isoforms were screened to identify specific PKC isoforms as potential targets for radiosensitization. Antisense oligonucleotide inhibition of PKC  $\delta$  and PKC  $\zeta$  significantly impaired the survival of radiation treated breast tumor cells (refer to July, 2000 annual report). In the absence of radiation insult and in the presence of low dose (1.5 Gy) radiation treatment, inhibition of PKC  $\delta$  attenuated breast tumor cell survival as well. Further supporting these findings, inhibition of PKC  $\delta$  activity by rottlerin reduced the survival of MCF-7 and MDA-MB-231 cells. Task 4 was completed in the June 2001-June 2002 funded year by experiments utilizing dominant negative PKC  $\delta$  and PKC  $\zeta$  plasmid constructs (generously provided by Dr. Weinstein, Columbia University, NY). MCF-7 cells with stable expression of PKC dominant negatives were selected for on the basis of neomycin resistance. MCF-7 cells transformed with PKC  $\delta$  or PKC  $\zeta$  dominant negative mutants demonstrated approximately 60% fewer viable cells than those transformed with the empty vector alone (Figure 1A; data not shown). Western blot analysis revealed that the levels of immunodetectable PKC  $\delta$  were elevated 3.5-fold in MCF-7 cells treated with the PKC  $\delta$  dominant negative (PKC $\delta$  dn) versus the empty plasmid vector, consistent with expression of mutant PKC  $\delta$  from the dominant negative (Figure 1B). Task 4- complete.

Task 5 defined an effective end-point assay for the survival of radiation and PKC antisense oligonucleotide treated cells. Clonogenic survival by the crystal violet staining method and the 96-well plate based MTS assay were compared (refer to July, 2001 annual report). The MTS assay was determined to be a rapid and reproducible assay for the measurement of relative cell survival and was used for subsequent experiments. Task 5- complete.

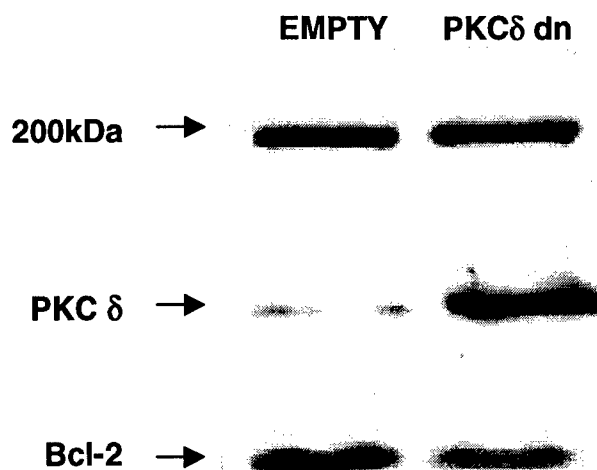
Task 6 proposed to measure DNA damage in response to radiation and PKC oligonucleotides. The comet assay performed under alkali conditions permitted sensitive and quantitative detection of single- and double-strand DNA breaks. Tail moment is a measure of the relative intensity and electrophoretic mobility of fragmented nuclear DNA. Tail moments less than 2 are indicative of undamaged DNA while those greater than 2 indicate DNA damage. The majority (60%) of comets for control MDA-MB-231 cells had tail moments less than 2 (Figure 2; appended manuscript Fig.6, McCracken et al.). Treatment of MDA-MB-231 cells with PKC  $\delta$  antisense oligonucleotide or 1.5 Gy radiation caused a significant shift in the comet distribution toward those with tail moments greater than 2 consistent with DNA damage. The combination of PKC  $\delta$  antisense oligonucleotide and radiation produced a further increase in damaged DNA. Depletion of PKC  $\delta$  by antisense oligonucleotide resulted in a loss of DNA integrity. Task 6- complete.



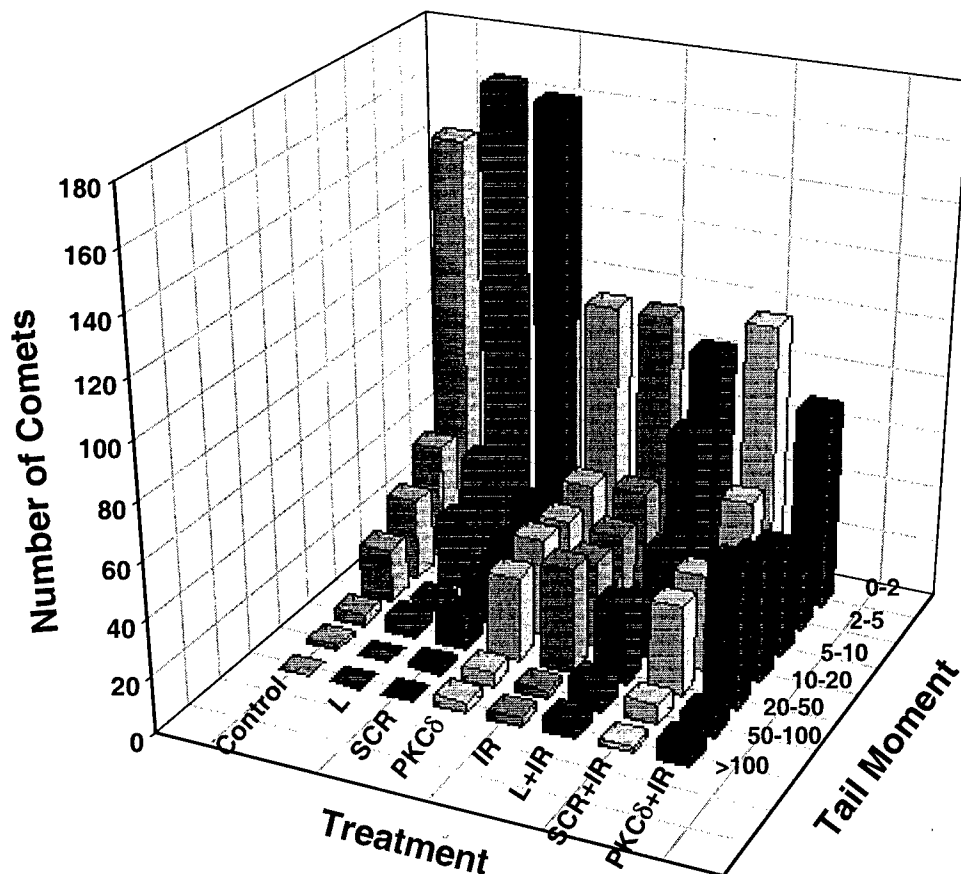
(A)



(B)



**Figure 1. MCF-7 cell transformation with PKC  $\delta$  dominant negatives.** MCF-7 cells ( $1 \times 10^6/35 \text{ mm}^2$  dish) were transfected with  $10 \mu\text{g}$  pcDNA3-neo (EMPTY) or  $13.7 \mu\text{g}$  pcDNA3-neo-PKC $\delta$  dn (normalized for neomycin resistance gene copy number). Post-transfection (96 h) culture medium was exchanged with DMEM/10% FBS containing  $400 \mu\text{g/ml}$  G418. A, After 11 days of growth in selection medium cells were harvested and counted using a hemocytometer. B, After 11 days of growth in selection medium whole cell extracts were prepared. Proteins ( $30 \mu\text{g/lane}$ ) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with a PKC  $\delta$  and Bcl-2 antibodies. A 200KDa band that reacts with PKC  $\delta$  antibody is shown as a loading control. Data are the mean  $\pm$  SE of  $n=3$  experiments and statistically significant differences are indicated \* ( $P<0.05$ ) (A). Blot is typical of  $n=3$  experiments (B).



**Figure 2. Comet analysis of MDA-MB-231 cells treated with PKC  $\delta$  oligonucleotide +/- radiation.** MDA-MB-231 cells ( $1.1 \times 10^5/35$  mm<sup>2</sup> dish) were treated with nothing (control), lipofectin (L), scrambled oligonucleotide (SCR) or PKC  $\delta$  oligonucleotide (PKC $\delta$ ). Following transfection (24 h), cells were irradiated (1.5 Gy IR) and prepared for comet analysis. The number of comets with tail moments in ranges between 0-2, 5-10, 10-20, 20-50, 50-100 and >100 are plotted for each treatment group. Data represent n=3 independent experiments with 80 comets scored per treatment/experiment. Statistically significant differences are indicated below (\*P<0.05).

#### Statistical Analyses (ANOVA)

Comparison	P Value
*Control, L and SCR vs. PKC $\delta$	<0.05
*Control, L, SCR and PKC $\delta$ vs. IR, L+IR, SCR+IR and PKC $\delta$ +IR	<0.05
*IR and SCR+IR vs. PKC $\delta$ +IR	<0.05

## KEY RESEARCH ACCOMPLISHMENTS:

- Optimization of liposome-mediated delivery of plasmids and antisense oligonucleotides was accomplished. (Task 1)
- Treatment of MCF-7 and MDA-MB-231 cells with PKC  $\delta$  and PKC  $\zeta$  antisense oligonucleotides resulted in significant reductions of PKC  $\delta$  and PKC  $\zeta$  protein levels. (Task 3)
- PKC  $\delta$  antisense oligonucleotide reduced MDA-MB-231 cell survival in the absence and in the presence of high (5.6 Gy) and low (1.5 Gy) dose radiation treatments. Task 4
- PKC  $\delta$  antisense oligonucleotide reduced MCF-7 cell survival in the presence of high dose radiation treatment. Task 4
- PKC  $\zeta$  antisense oligonucleotide reduced the survival of MCF-7 and MDA-MB-231 cells treated with high dose radiation. Task 4
- Depletion of PKC  $\delta$  from MDA-MB-231 cells by PKC  $\delta$  antisense oligonucleotide resulted in a loss of DNA integrity; Depletion of PKC  $\zeta$  by PKC  $\zeta$  antisense oligonucleotide did not. Task 6
- Transformation of MCF-7 cells with PKC  $\delta$  and PKC  $\zeta$  dominant negative plasmid constructs reduced cell survival. Task 4
- The PKC  $\delta$  inhibitor, rottlerin reduced MCF-7 and MDA-MB-231 cell survival. Task 4
- The MTS assay was defined as a suitable end-point for the assessment of breast tumor cell survival following radiation and/or PKC oligonucleotide treatment(s). Task 5

## REPORTABLE OUTCOMES:

- June, 2002      Principal Investigator nominated as Senior Research Fellow at the University of Michigan, Radiation Oncology Department.
- May, 2002      Principal Investigator awarded Ph.D. from West Virginia University in Genetics and Developmental Biology.
- July, 2001      Principal Investigator granted attendance to the American Association for Cancer Research, "Pathobiology of Cancer Workshop" in Keystone, CO.

## Publications and Abstracts

**McCracken MA**, Miraglia LJ, McKay RA and Strobl JS: PKC delta is a pro-survival factor in human breast tumor cell lines. Manuscript in preparation- to be resubmitted to *The International Journal of Cancer* by August 15, 2002.

Zhou Q, **McCracken MA** and Strobl JS: Control of mammary tumor cell growth *in vitro* by novel cell differentiation and apoptosis agents. *Breast Cancer Research and Treatment*, In Press.

Liu C, **McCracken MA**, Strobl JS, Schilling JK and Kingston DGI: Design and synthesis of steroid linked taxol analogs: A new route toward selective delivery of cytotoxic taxanes. 43<sup>rd</sup> Annual Meeting of the American Society of Pharmacognosy and 3<sup>rd</sup> Monroe Wall Symposium, 2002.

Martirosyan A, Zhou Q, Bata R, **McCracken M**, Freeman A, Morato-Lara C and Strobl JS: New pharmacologic agents promote cell differentiation in human breast tumor cells. *Proc. of the 41<sup>st</sup> Annual American Society for Cell Biology Meeting*, Abstract#762, 2001.

Melkounian Z, **McCracken MA** and Strobl JS: Suppression of *c-myc* protein and induction of cellular differentiation in human breast cancer cells but not in normal human breast epithelial cells by quinidine. *Proc. of the 41<sup>st</sup> Annual American Society for Cell Biology Meeting*, Abstract#70, 2001.

Gahr S, Kocamis H, **McCracken MA** and Killefer J: The expression of TGF-beta family members in MCF-7 and MDA-MB-231 breast tumor cell lines. *Proc. Experimental Biology*, 2001.

**McCracken MA**, McKay RA, and Strobl JS: Protein kinase C delta involvement in radiation-induced mammary tumor cell death. *Proc. of the 92<sup>nd</sup> American Assoc. Cancer Res.*, Vol. 42:Abstract# 3583, 2001.

**McCracken MA**, Miraglia L and Strobl JS: The effects of protein kinase C antisense oligonucleotides on mammary tumor cell lines. *Proc. of the 91<sup>st</sup> American Assoc. Cancer Res.*, Vol. 41:Abstract# 4491, 2000.

**McCracken MA:** Combination radiation and antisense oligonucleotide gene therapy for breast cancer. *Proc. Susan G. Komen Foundation Meeting*, 2000.

**McCracken MA,** Miraglia L and Strobl JS: Sensitization of breast cancer cells to ionizing radiation by protein kinase C inhibition. *Proc. of the 90<sup>th</sup> American Assoc. Cancer Res.*, Vol. 40:Abstract# 4216, 1999.

**List of Personnel Receiving Pay:**

Dr. Meredith A. McCracken, Post-doctoral Research Fellow, Department of Physiology and Toxicology at West Virginia University

**Conclusions:**

The goal of this project was to identify individual PKC isoforms that function in the survival of radiation treated breast tumor cells. The major finding from this project was that PKC  $\delta$  is a survival factor in human breast tumor cells. PKC  $\delta$  inhibition not only inhibited the survival of radiation treated breast tumor cells but inhibited breast tumor cell survival independent of radiation treatment. These results imply that PKC  $\delta$  is a general survival factor in breast tumor cells and support further research into the development of novel therapeutic strategies targeted to the  $\delta$  isoform of protein kinase C.

All proposed tasks and objectives were successfully completed.

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Begemann M, Kashimawo SA, Hietjan DF, Schiff PB, Bruce JN and Weinstein IB (1998) Treatment of human glioblastoma cells with the staurosporine derivative CGP 41251 inhibits CDC2 and CDK2 kinase activity and increases radiation sensitivity. *Anticancer Res* **4A**:2275-2282.

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Hallahan DE, Virudachalam S, Schwartz JL, Panje N, Mustafi R and Weichselbaum RR (1992) Inhibition of protein kinase C sensitizes human tumor cells to ionizing radiation. *Radiat Res* **129**:345-350.

O'Brian C, Vogel VG, Singletary SE and Ward NE (1989) Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. *Cancer Res* **49**:3215-3217.

Soh J-W, Lee EH, Prywes R and Weinstein IB (1999) Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Mol Cell Biol* **19**:1313-1324.

Zhang W, Yamada H, Sakai N and Nozawa Y (1993) Sensitization of C6 glioma cells to radiation by staurosporine, a potent protein kinase C inhibitor. *J Neurooncol* **15**:1-7.



Report

## Control of mammary tumor cell growth *in vitro* by novel cell differentiation and apoptosis agents

Qun Zhou<sup>1</sup>, Meredith A. McCracken<sup>2</sup>, and Jeannine S. Strobl<sup>1,2</sup>

<sup>1</sup>Department of Biochemistry and Molecular Pharmacology, <sup>2</sup>Genetics and Developmental Biology Program, West Virginia University, Morgantown, WV, 26506, USA

**Key words:** apoptosis, breast cancer, cell differentiation agents, chloroquine, MCF-7, quinidine, quinine

### Summary

The use of breast tumor differentiating agents to complement existing therapies has the potential to improve breast cancer treatment. Previously we showed quinidine caused MCF-7 cells to synchronously arrest in G1 phase of the cell cycle, transition into G0 and undergo progressive differentiation. After 72–96 h cells became visibly apoptotic. Using several analogs of quinidine we determined that MCF-7 cell cycle exit and differentiation are typical of quinoline antimalarial drugs bearing a tertiary amine side chain (chloroquine, quinine, quinidine). Differentiated cells accumulated lipid droplets and mammary fat globule membrane protein. Apoptosis was assayed by a nucleosome release ELISA. Quinidine and chloroquine triggered apoptosis, but not quinine, a quinidine stereoisomer that displayed weak DNA binding. The apoptotic response to quinidine and chloroquine was p53-dependent. A 4–15-fold induction of p21(WAF1) protein was observed in cells treated with quinidine or chloroquine prior to apoptosis, but p21(WAF1) was not increased in cells that differentiated in response to quinine. Chloroquine was most active in stimulating MCF-7 apoptosis, and quinine was most active in promoting MCF-7 cell differentiation. We conclude, distinct mechanisms are responsible for breast tumor cell differentiation and activation of apoptosis by quinoline antimalarials. Alkylamino-substituted quinoline ring compounds represented by quinidine, quinine, and chloroquine will be useful model compounds in the search for more active breast tumor differentiating agents.

### Introduction

Our laboratory is investigating the use of quinoline antimalarials as inexpensive drugs with low toxicity for adjuvant breast cancer treatment. Quinidine is a natural alkaloid that is derived from the bark of the cinchona tree. Quinidine is used therapeutically to treat cardiac arrhythmia and malaria. Quinine is present in cinchona tree bark in even higher concentrations than quinidine. Quinine has antimalarial activity equivalent to that of quinidine but is not used as an antiarrhythmic agent [1]. Interestingly, quinidine and quinine are stereoisomers (Figure 1). In quinidine, the secondary alcohol of the side-chain in the 4-position of the quinoline ring is the dextrorotary conformation while in quinine the 4-hydroxyl group exists in the levorotatory conformation. The synthetic

alkaloid, chloroquine is a more potent antimalarial than either quinidine or quinine. Chloroquine is a 4-alkylamino substituted quinoline that also possesses a chlorine substitution at the 7-position of the ring. Despite chloroquine's structural similarities with quinine and quinidine, chloroquine has a different mechanism of antimalarial activity, and chloroquine-resistant malaria responds to quinine treatment. The quinoline ring by itself lacks antimalarial or antiarrhythmic activity indicating that therapeutic properties are conferred by the side chain substituents. This group of pharmacologically active compounds might have anticancer properties.

We reported previously that quinidine causes moderate growth arrest and morphologic differentiation of human breast cancer cells *in vitro* [2–4]. In an experiment which compared MCF-7 cell numbers after 5

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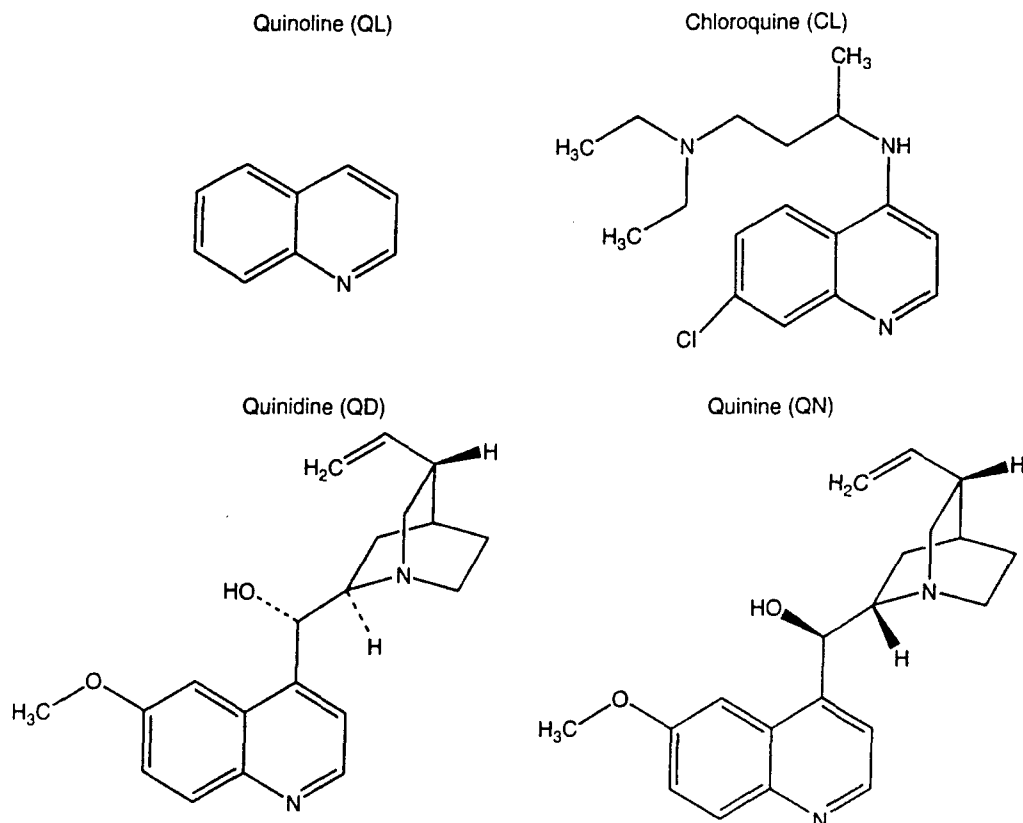


Figure 1. Structures of antimalarials.

days growth in the presence and absence of quinidine, 25  $\mu\text{M}$  quinidine was found to reduce the increase in cell numbers by 50% [2]. Quinidine promoted cell cycle arrest in G1, exit into G0 marked by a loss of expression of Ki67 antigen, and lipid droplet accumulation and cytoplasmic enlargement, morphological evidence of cellular differentiation [3, 4]. Accumulation of MCF-7 cells in the G1/G0 phase of the cell cycle was maximal between 24 and 48 h with 90  $\mu\text{M}$  quinidine. The potassium channel blocking activity of quinidine is implicated in the G1 arrest of MCF-7 cells, although the signaling pathway has not yet been elucidated [3]. The mechanism of quinidine action on growth in MCF-7 cells involves a number of changes in cell cycle proteins that regulate progression through G1 phase [4, 5]. Quinidine (90  $\mu\text{M}$ ) treatment caused increased p21(WAF1), p53, and p27 protein levels, and decreased cyclin D1, phosphorylated pRb, and Myc. Quinidine also raised levels of acetylated histone H4, a response that has been correlated with cellular differentiation in breast tumor cells [4, 6]. The differentiation response to quinidine in MCF-7 cells was well developed by 48–72 h. In cells continuously

exposed to quinidine for 72–96 h, apoptotic nuclei stained with Hoechst dye were apparent [3], suggesting that quinidine causes both growth arrest, via cell cycle arrest and differentiation, and cell death.

The present report describes the results of a comparative study of the effects of quinidine, quinoline and two additional quinoline antimalarials, quinine and chloroquine on MCF-7 cell apoptosis and differentiation. Our data show that quinoline antimalarials inhibit growth of human breast cancer cells *in vitro* and support the hypothesis that quinoline antimalarials cause cellular differentiation and apoptosis via distinct pathways.

## Materials and methods

### Chemicals

Chloroquine (CL), Oil Red O (ORO), quinidine (QD), quinine (QN), quinoline (QL) and trichostatin A (TSA) were purchased from Sigma Chemical Company (St. Louis, MO).

### Tissue culture

*italics* ✓ Permanent cell lines derived from patients with breast carcinoma were used in these studies. MCF-7 cells between passage numbers 30–50, MCF-7<sup>tr</sup><sub>as</sub>, T47D and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah), and 40 µg/ml gentamicin. Experiments were performed in DMEM/5%FBS. The cells were cultured at 37°C in a humidified atmosphere of 93% air/7% CO<sub>2</sub>.

*must present* MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay

MCF-7 cells were plated at  $4.0 \times 10^3$  cells/well in 96-well plates in 225 µl of DMEM/5% FBS. Twelve hours after plating, the test agents were added and the cells were incubated for an additional 48 h. Cell growth was measured using a MTS assay kit (Cell-Titer 96 AQueous one solution assay, Promega, Madison, WI) according to the manufacturer's instructions. Assays were repeated at least three times. The concentration of each agent that inhibited cell growth by 50% (IC<sub>50</sub>) was determined using non-linear regression analysis to fit the inhibition data (Prism 3.0, GraphPad Software, Inc., San Diego, CA).

### Ki67 immunohistochemical assay

An immunohistochemical assay for Ki67 antigen was performed according to the protocol described by Wang et al. [3]. MCF-7 cells ( $2 \times 10^5$ /dish) were plated on ethanol-washed glass coverslips in 35 mm<sup>2</sup> dishes. Twelve hours after plating, test compounds were added to the medium using the IC<sub>50</sub> determined in the MTS assay. Forty-eight hours later, the cells were fixed in acetone:ethanol (50:50) on ice for 10 min, and washed with PBS-0.15% bovine serum albumin (BSA, fraction V, Sigma) (PBS-BSA). Cells were incubated for 10 min with 0.3% hydrogen peroxide in methanol, rinsed with PBS-BSA, and incubated for 30 min with 10% horse serum in PBS-BSA. Cells were incubated for 60 min with the primary antibody Ki67 (MIB-1, Dako Corporation, Glostrup, Denmark) diluted in PBS-BSA (1:77). After rinsing with PBS-BSA, the secondary antibody,

biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted in PBS-BSA (1:250) was added for 30 min. The cells were rinsed with PBS-BSA and incubated for 30 min with the avidin-biotin-peroxidase reagent (Vector Laboratories). After rinsing with PBS, the antigen-antibody complexes were visualized using diaminobenzidine (Stable DAB, Research Genetics, Huntsville, AL). The cells were counterstained with Mayer's hematoxylin (Fisher Scientific, Pittsburgh, PA), and the coverslips mounted using Permount (Fisher). Ki67 negative cells were visualized by light microscopy (400× objective, Ortholux microscope, Ernst Leitz, Wetzlar, Germany). The percentage of Ki67 negative cells in a population of at least 500 cells per experimental condition was determined.

### Oil red O assay

Lipid droplet accumulation in the cytoplasm was measured using Oil Red O (ORO) staining [7]. MCF-7 cells ( $1 \times 10^5$  per dish) were plated on ethanol-washed glass coverslips in 35 mm<sup>2</sup> dishes. Cells were treated with drug or vehicle for 48–72 h, fixed in 10% formaldehyde-0.2% calcium acetate in PBS for 3 min and stained for 10 min using the ORO stock solution (0.5% ORO in 98% isopropanol) diluted 6:4 in distilled water. The coverslips were rinsed in tap water, counterstained with Mayer's Hematoxylin solution, and mounted using 50:50 (v/v) glycerol/water. Lipid droplet accumulation was visualized by light microscopy (400×). Positive ORO cells had at least 10 lipid droplets per cell. The percentage of positive ORO cells was determined by counting at least 300 cells per experimental condition.

### Western blotting

Cells were harvested from confluent T-75 flasks and subcultured ( $1 \times 10^6$ ) in 100 mm<sup>2</sup> dishes. Cell lysates were prepared by scraping cells into ice-cold harvesting buffer (1% SDS-10 mM Tris-HCl, pH 7.4). The lysates were boiled for 5 min, and protease inhibitors added (Protease Inhibitor Mixture, Roche Applied Sciences, Indianapolis, IN). The supernatants were collected after centrifugation in an Eppendorf micro-centrifuge (14,000 rpm, 5 min) at 4°C. The protein concentration of the supernatant was determined using a BCA protein assay (Pierce, Rockford, IL) and BSA as a standard. Equal amounts of protein were loaded onto 12% SDS-polyacrylamide mini-gels. Colored

molecular weight protein markers (Amersham Pharmacia Biotech Inc., Piscataway, NJ) were used to estimate the molecular weight of the immunoreactive proteins. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Invitrogen, Carlsbad, CA) and blocked overnight at 4°C using 3% non-fat milk blocking buffer (3 g non-fat dry milk per 100 ml of TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and 0.05% (v/v) Tween 20). Membranes were incubated for 3 h at room temperature with the following primary antibodies: mouse monoclonal anti-p21 (WAF1) (Ap-1), mouse monoclonal anti-p53 (Ap-5) (Oncogene, Cambridge, MA) or mouse anti-human milk fat globule membrane (MFGM) protein (MAB-4043, Chemicon International, Temecula, CA). The primary antibodies were diluted 1:500 in Western washing solution (0.1% non-fat dry milk, 0.1% albumin (chicken egg), 1% (v/v) FBS, 10% (v/v) of 10 × PBS, pH 7.3, 0.2% (v/v) Tween-20). After washing three times with Western washing solution and one time with TBS, the antigen-antibody complexes were incubated 1 h at room temperature with HRP (horseradish peroxidase)-conjugated secondary antibody (anti-mouse IgG-HRP, Santa Cruz Biotechnology) diluted 1:3000 in Western washing solution. After washing three times with TBS, antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography.

#### Densitometric analysis

Autoradiograms of the Western immunoblots were scanned using Chemilmager software (Alpha Innotech Corporation, San Leandro, CA). The blots were adjusted for brightness and contrast, and the mean density for each band was analyzed using Chemilmager analysis program. The background value was subtracted from each individual object.

#### Cell death ELISA (enzyme-linked immunosorbent assay)

Apoptotic cells were measured using the cell death ELISA kit (Roche Applied Science). Cells ( $4.0 \times 10^3$ ) were plated in each well of 96-well plates and treated in triplicate with either drug or vehicle (control cells) for 48 or 72 h. Cell cytoplasmic fractions were prepared and 20 µl aliquots were transferred into streptavidin-coated microtiter plates (MTP) for analysis as per the instructions of the supplier. Apoptosis, measured as nucleosome release into the

cytoplasmic fraction, was quantified spectrophotometrically (A<sub>405</sub> nm, EL340 Biokinetics Reader, Bio-Tek Instruments, Winooski, Vermont) using ABTS (2,2-Azino-di-3-ethylbenzthiazolin-sulfonate) as the substrate.

#### P21(WAF1) ELISA

Cells were plated ( $1 \times 10^6$  cells/100 mm<sup>2</sup> dish); 12 h later MTS IC50 of each test agent were added to the medium and incubated for 24 h. Fifty micrograms protein aliquots of the cell lysates were assayed for p21(WAF1) using a colorimetric ELISA (Oncogene, Boston, MA) according to the manufacturer's instructions.

#### DNA binding assay

Chloroquine, quinidine, and quinine emit an intense blue fluorescence when excited by ultraviolet light [8]. Intercalation into DNA quenches the chloroquine fluorescence [9], and this principle is the basis of the fluorescence assay we used to assess DNA binding by quinidine and quinine. Stock solutions of chloroquine, quinidine, and quinine in water were diluted 1:1 in 10 mM Tris, 1 mM EDTA, pH 8 (TE) or TE containing 1 µg of lambda DNA (Invitrogen, Carlsbad, CA) to a final concentration of 100 µg drug/ml. The final reaction volume was 20 µl. Drugs or drugs plus DNA were incubated for 15 min at room temperature in the dark in a 96-well plate (Nunc Immunoplate, Nunc Nalge International). Fluorescence was then measured using a CytoFluor4000 (PerSeptive Biosystems) instrument using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Background fluorescence from TE alone or TE plus DNA samples was subtracted from each measurement as appropriate. Data were expressed as the fluorescence quench ratio defined as the average fluorescence of drug alone (Em1) divided by that of drug in the presence of DNA (Em2).

#### Statistical analysis

Data are expressed as the mean ± SE for *n* number of replicates as indicated in the figure legends. One-way ANOVA (analysis of variance) followed by Bonferroni's *t*-tests were used to assess statistically significant differences between control- and drug-treated groups (GraphPad Instat, Intuitive Software for Science, San Diego, CA).

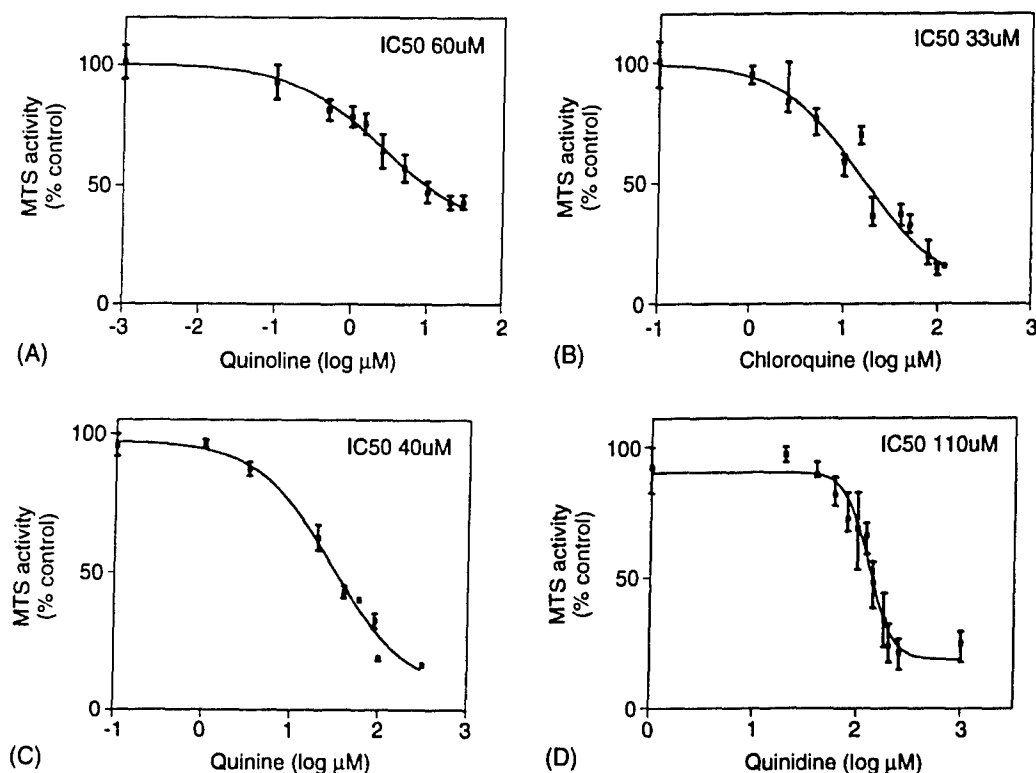


Figure 2. Antimalarials inhibited MTS metabolism in MCF-7 cells. MCF-7 cells (4000 cells/well) were grown in 96 well plates in the presence of increasing concentrations of each antimalarial for 48 h. Cell growth was estimated using the MTS assay. The  $\text{IC}_{50}$  value for each agent was determined using nonlinear regression analysis to fit inhibition data. Data are the mean  $\pm$  SE of three independent experiments performed in quadruplicate. MTS activity of untreated cells was set 100%. The  $\text{IC}_{50}$  value for each antimalarial is shown in each panel.

## Results

### *Antimalarials inhibit growth and promote MCF-7 cell differentiation*

Quinidine inhibits MCF-7 cell proliferation, and to test whether this is a general response to structurally related chemicals, the effects of quinidine, quinine, chloroquine, and quinoline on MCF-7 cells on cell growth were compared. The MTS assay directly measures mitochondrial metabolic activity; none of the test agents directly inhibited mitochondrial metabolism of MTS after a 2 h or a 6 h exposure (data not shown). Therefore, MTS activity measured after 48 h incubation with these compounds is a valid measure of the number of surviving cells (Figure 2). The  $\text{IC}_{50}$  was estimated for each drug in the MTS assay, and the order of potency of the compounds was chloroquine ( $\text{IC}_{50}$ , 33  $\mu\text{M}$ ) > quinine ( $\text{IC}_{50}$ , 40  $\mu\text{M}$ ) > quinoline ( $\text{IC}_{50}$ , 62  $\mu\text{M}$ ) > quinidine ( $\text{IC}_{50}$ , 110  $\mu\text{M}$ ). The effects of chloroquine, quinine, quinoline, and quinidine on cell numbers were investigated using the  $\text{IC}_{50}$  values determined in the MTS assay. Chloroquine ( $\text{IC}_{50}$ ,

33  $\mu\text{M}$ ) caused a  $\sim 60\%$  decrease in cell numbers after a 60 h incubation as compared with control, growing MCF-7 cells suggesting that chloroquine caused cell death. In parallel cell cultures incubated with MTS  $\text{IC}_{50}$  of quinoline, quinidine, and quinine, the cell numbers after 60 h did not differ from the plating density. These compounds may primarily arrest cell growth, or alternatively permit a limited amount of proliferation balanced by cell loss (Figure 3).

Earlier studies showed quinidine caused G1 arrest and exit from the cell cycle [3]. Ki67 is a nuclear protein that is expressed throughout the cell cycle. The absence of Ki67 protein is a marker for non-proliferating cells that have entered G0 phase, and, therefore, provides a means of monitoring whether cell cycle exit might contribute to growth arrest [10]. By measuring Ki67 expression immunohistochemically, chloroquine (33  $\mu\text{M}$ ), quinidine (110  $\mu\text{M}$ ), and quinine (40  $\mu\text{M}$ ) were shown to promote exit from the cell cycle by 48 h. Under normal culture conditions, 95% of MCF-7 cells are engaged in the cell cycle, and express Ki67 antigen; 5% of the cells in the control population were negative for immunore-

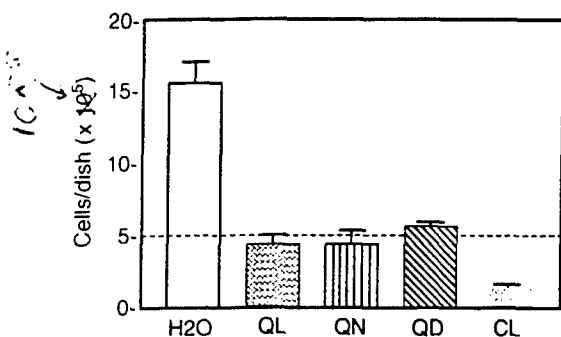


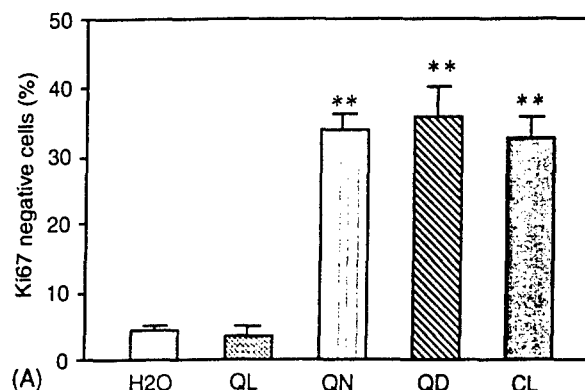
Figure 3. Effects of antimalarials on cell growth. MCF-7 cells ( $5 \times 10^5$ ) growing in  $60 \text{ mm}^2$  dishes in DMEM/5%FBS were treated with concentrations of antimalarials corresponding to 50% inhibition of MTS activity as measured at 48 h. After 60 h incubation with antimalarials or solvent alone, viable cells that excluded trypan blue were counted using hemacytometer. Data are the mean of  $n = 3 \pm \text{SE}$  independent experiments.

active Ki67 (Figure 4(A)). The percentage of Ki67 negative MCF-7 cells after 48 h growth in the presence of chloroquine, quinidine or quinine increased 6–7-fold to 30–40% compared to control cells ( $p < 0.01$ ) (Figure 4(A)). Thus, growth inhibition by chloroquine, quinine, and quinidine can be explained in part by exit of cells from the cell cycle. Quinoline (62  $\mu\text{M}$ ), however, caused cell numbers to stabilize without shifting cells into G0.

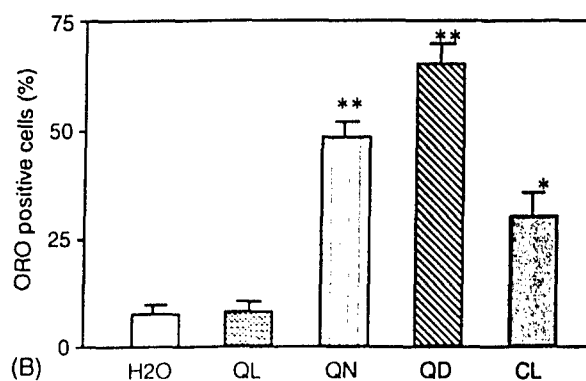
Quinine, chloroquine, and quinidine also were similar in their ability to promote a more differentiated phenotype in MCF-7 cells, the accumulation of ORO positive lipid droplets in the cytoplasm [11] (Figure 4(B)). Quinoline was inactive in this assay as well. The data suggest that the stereoisomers, quinidine, and quinine act similarly, but with different potency, causing growth arrest, exit from the cell cycle and differentiation. Chloroquine was more potent and more toxic than quinidine and quinine. Chloroquine caused cell cycle exit; examination of the cells after ORO staining revealed morphologic evidence of differentiation (lipid droplets) as well as apoptotic cell death (condensed cells with heavy nuclear staining with hematoxylin). Quinoline caused arrest of cell growth without promoting cell cycle exit or any evidence of differentiation. Quinoline-treated cells showed no evidence of cytotoxicity, and the mechanism for the growth arrest is unclear.

In breast cells, the human milk fat protein is another cell differentiation marker [12]. The monoclonal antibody MFGM identifies antigens found on the milk fat globule membrane, which surrounds milk fat [13]. The expression of MFGM protein was measured by

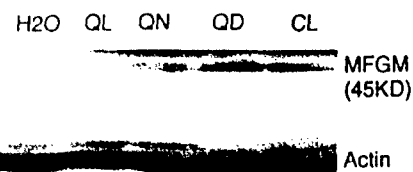
western blot analysis in MCF-7 cells exposed to antimalarials for 48 h. The densitometric signals in arbitrary units derived from scanning the immunoblots



(A)



(B)



(C)

Figure 4. Cell differentiation by antimalarials. Ki67 expression, lipid droplet accumulation and human MFGM levels were measured in MCF-7 cells. MCF-7 cells were grown in  $35 \text{ mm}^2$  dishes on sterile glass coverslips in DMEM/5%FBS. Cells were treated with solvent (distilled  $\text{H}_2\text{O}$ ) or the MTS  $\text{IC}_{50}$  of each antimalarial for 48 h (A) Ki67 immunoreactivity was performed as previously described by Wang et al. [3]. The data represent the mean percentage of Ki67 negative cells in each treatment group ( $n = 3 \pm \text{SE}$ ); 500 cells per experiment were counted. (B) Lipid droplet accumulation was measured using ORO staining in MCF-7 cells treated with antimalarials. The data represent the percentage ORO positive cells in each treatment group ( $n = 3 \pm \text{SE}$ ); 300 cells per treatment were counted. (C) Protein aliquots (25  $\mu\text{g}$ ) from the cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and analyzed by western blotting using an antibody specific for MFGM. Actin protein was used as a loading control. Data are representative of two independent experiments. Statistically significant differences between control and treatment groups are indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

is provided in parantheses following each treatment. Quinidine (8872), quinine (7548), and chloroquine (8205) increased MFGM protein compared with control (5241) MCF-7 cells (Figure 4(C)). In contrast, MFGM protein levels were not changed by quinoline (5312) treatment. Changes of MFGM in MCF-7 cells by quinidine, quinine, and chloroquine are consistent with induction of lipid droplets. The coinduction of ~~with~~ fat, and lipid droplets support our conclusion that quinidine, quinine, and chloroquine caused differentiation in MCF-7 cells.

#### Effect of antimalarials on apoptosis in MCF-7 cells

Morphological evidence that quinidine activated apoptosis in MCF-7 cells [3] prompted examination of apoptosis in cells exposed to antimalarials using the nucleosome release assay. Levels of apoptosis after 48 and 72 h were measured over a range of concentrations that spanned the respective MTS IC<sub>50</sub> values for each compound. Etoposide (30  $\mu$ M), a topoisomerase II inhibitor, elicits nucleosomal laddering in MCF-7 cells and was used as a positive control for this assay [14]. The enrichment of apoptotic response, defined as the ratio of the apoptosis signal in the presence and absence of MTS IC<sub>50</sub> concentrations of antimalarials, calculated after 48 and 72 h is summarized in Table 1. The concentration-response curve measured for each compound at 72 h is shown in Figure 5.

Quinidine and chloroquine stimulated nucleosome release in MCF-7 cells in a concentration-dependent fashion ( $p < 0.05$ ). Chloroquine was more potent than quinidine and apoptosis was more extensive in cells exposed to chloroquine than quinidine. In contrast,

quinoline, and quinine treatments did not change nucleosome release as compared with controls ( $p > 0.05$ ). Quinoline displayed incomplete inhibition of MTS activity, no differentiating activity, and was not expected to activate apoptosis. However quinine inhibited MTS metabolism nearly as potently as chloroquine, and stimulated cellular differentiation. The experiments provide evidence that apoptosis, measured by nucleosome release, is differentially stimulated by the stereoisomers, quinidine, and quinine, while exit from the cell cycle, differentiation, and antimalarial activity are all stereo non-selective responses.

To investigate the stereoselectivity of the apoptotic response further, levels of p53 and a downstream target of p53, p21(WAF1), were measured in MCF-7 cells exposed for 24 h to quinidine, quinine, chloroquine or quinoline. At their MTS IC<sub>50</sub> levels, quinidine and chloroquine elevated p53 protein in MCF-7 cells, but in cells exposed to quinine and quinoline, p53 was undetectable (Figure 6(A)). Protein p21(WAF1) was increased in chloroquine and quinidine treated cells, but not in quinine or quinoline treated cells (Figure 6(B)). An ELISA was performed on MCF-7 whole cell extracts to quantify the changes in p21(WAF1) protein in response to the antimalarials (Figure 7(A)). Trichostatin acid (TSA) was used as a positive control for these experiments. Transcription of p21(WAF1) and p21(WAF1) protein levels have been shown to be increased by TSA [15, 16]. Chloroquine caused a 10–15-fold elevation in p21(WAF1) levels in MCF-7 cells at 24 h (Figure 7(B)). The p21(WAF1) response to chloroquine exceeded that of the potent histone deacetylase inhibitor, TSA. Quinidine elevated p21(WAF1) levels 4–5-fold, approximately the same as TSA, but neither quinine nor quinoline raised p21(WAF1) protein.

Moreover, p21(WAF1) protein expression in response to quinidine (90  $\mu$ M) was different in wild type p53 and mutant p53 human breast cancer cells. Quinidine treatment increased p21(WAF1) protein in MCF-7 and MCF-7ras cells (wild type p53) (Figure 8), but p21(WAF1) protein was not detectable in malignant MDA-MB-231 and T47D cells (mutant p53) using western blot analysis. We previously reported that quinidine (90  $\mu$ M) induced cellular differentiation in all of these lines of breast cancer cells [4].

#### DNA binding by antimalarials

Chloroquine intercalates into DNA [9, 17, 18], but DNA intercalation is not required for antimalarial

Table 1. The apoptosis enrichment factor was calculated using data obtained from ELISA, and is the ratio: units of absorbance drug treatment group/units of absorbance control group

Compounds	Apoptosis enrichment factor (mean $\pm$ SE, $N = 3$ )	
	48 h	72 h
Quinoline (62 $\mu$ M)	1.00 $\pm$ 0.06	1.09 $\pm$ 0.03
Quinine (40 $\mu$ M)	1.13 $\pm$ 0.09	1.19 $\pm$ 0.07
Quinidine (110 $\mu$ M)	1.50 $\pm$ 0.10*	1.73 $\pm$ 0.09*
Chloroquine (33 $\mu$ M)	1.75 $\pm$ 0.14*	1.82 $\pm$ 0.07*
Etoposide (30 $\mu$ M)	2.74 $\pm$ 0.13*	1.23 $\pm$ 0.20
Trichostatin A (35 nM)	0.96 $\pm$ 0.06	0.91 $\pm$ 0.05

Results of treatment times of 48 h and 72 h are compared. Data shown are the mean  $\pm$  SE of three experiments performed in triplicate.

\*,  $p < 0.05$  for drug treatment versus control.

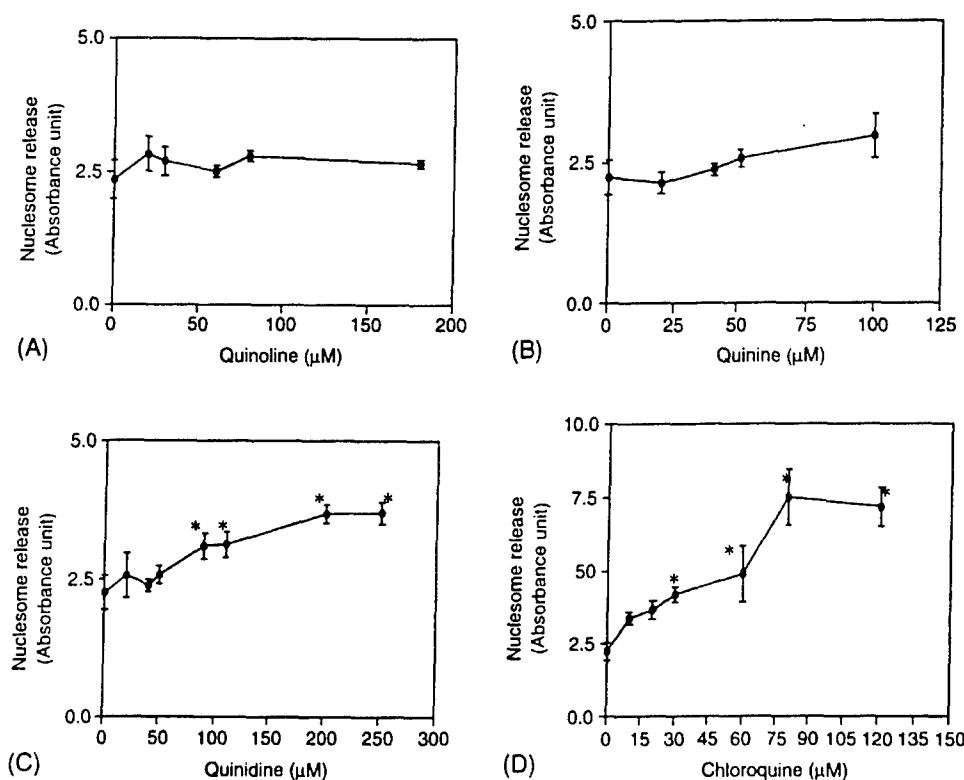


Figure 5. Nucleosome release apoptosis assay. MCF-7 cells (4000 cells/well) were grown in 96 well plates in the presence of increasing concentrations of antimalarials for 72 h. Nucleosome release was measured using the histone-DNA cell death detection kit as described in 'Materials and methods.' The level of nucleosomes released in each sample is indicated by the absorbance at 405 nm. Data are the mean  $\pm$  SE of three independent experiments performed in triplicate. Statistically significant differences between control and drug treatment groups at specific drug concentrations are indicated (\*,  $p < 0.05$ ). Note the change in scale of the x-axis in Panel D.

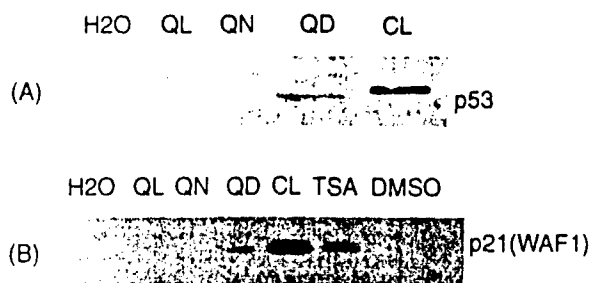
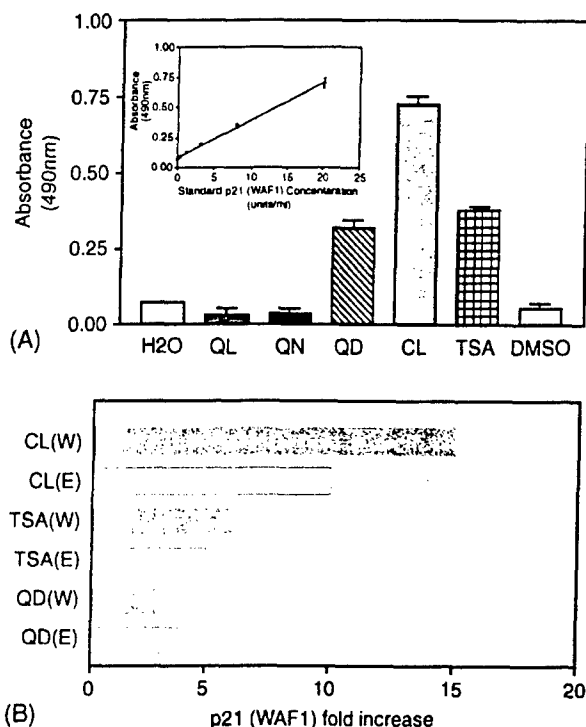


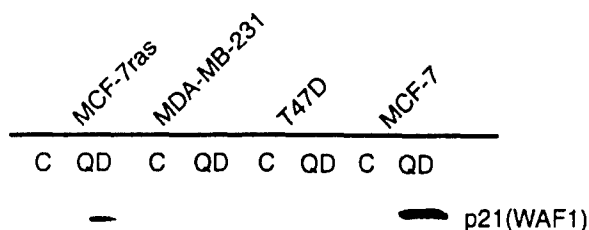
Figure 6. Western blot analysis of p21(WAF1) and p53 protein expression in human breast cancer cells. MCF-7 cells ( $1 \times 10^6$ ) were grown in 100 mm<sup>2</sup> dishes and treated with MTS IC<sub>50</sub> of antimalarials for 24 h. Protein aliquots (50  $\mu\text{g}$ ) from the cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and analyzed by western blotting using (A) mouse monoclonal antibody for p53. Data shown represent three independent experiments that showed the same results (B) mouse monoclonal antibody for p21(WAF1) (single experiment).

activity. We hypothesize that DNA intercalation is responsible for chloroquine-induced apoptosis, and that p53 induction by chloroquine is a consequence of DNA damage arising from intercalation. Our hypothesis predicts that chloroquine and quinidine but

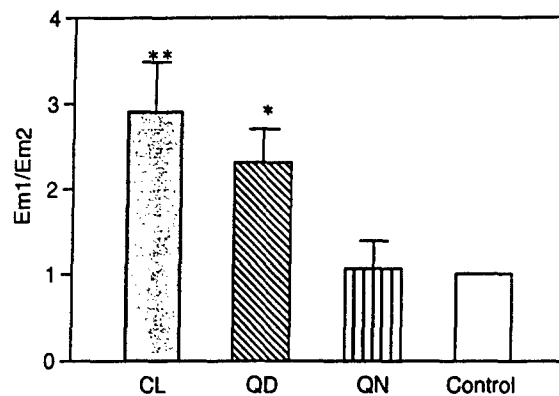
not quinoline and quinine intercalate into DNA. The binding of chloroquine, quinidine, and quinine to DNA was measured in an *in vitro* cell-free assay. The fluorescence signal from 2  $\mu\text{g}$  of free chloroquine, quinidine, and quinine dissolved in 10 mM Tris-1 mM EDTA, pH 8 was equivalent. The addition of lambda DNA to solutions of chloroquine and quinidine, but not quinine, caused a statistically significant fluorescence quenching (Figure 9). The average fluorescence quench ratio for chloroquine in four independent experiments was 2.9 while that for quinidine was 1.8. Quinine exhibited a fluorescence quench ratio of 1.1 which was not statistically significant. The results imply stereo-selective DNA binding by quinidine, and are consistent with the pattern of quinidine activation of nucleosome release, p53 and elevations in p21(WAF1) protein levels. This is the first report of quinidine binding to DNA. Using an assay that measured changes in plasmid superhelical density, quinine was reported to intercalate into DNA, however this binding is relatively weak compared to chloroquine [18].



**Figure 7.** Effect of antimalarials on p21(WAF1) protein expression in MCF-7 human breast cancer cells. (A) p21(WAF1) ELISA. MCF-7 cells ( $1 \times 10^6$ ) were plated in 100 mm<sup>2</sup> dishes in DMEM/5% FBS, treated with solvent (distilled-H<sub>2</sub>O or 0.01% DMSO) or MTS IC50 of each antimalarial for 24 h, and cell lysates prepared. TSA was dissolved in DMSO; antimalarials were dissolved in distilled H<sub>2</sub>O. Proteins (50 µg) from the cell lysates were assayed using p21(WAF1) ELISA kit as detailed in 'Materials and methods' using a 20 min incubation time. Data are the mean  $\pm$  SE of two independent experiments. (B) Comparison of p21(WAF1) protein changes detected in ELISA (E) and western blot analysis (W). W represents the fold increase in the signal density determined by densitometry; E represents the fold increase in absorbance in the ELISA. Fold increase is equal to drug treatment group divided by the control.



**Figure 8.** Effect of antimalarials on p21(WAF1) protein expression in human breast cancer cells. MCF-7, MDA-MB-231, T47D and MCF-7ras cells ( $1 \times 10^6$ ) were exposed to quinidine (90 µM) for 24 h. Protein aliquots (50 µg) from the cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and analyzed by western blotting using mouse monoclonal antibody for p21(WAF1) (single experiment).



**Figure 9.** Fluorescence quench assay for DNA binding activity. Changes in antimalarial fluorescence upon DNA binding were measured using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data were expressed as the fluorescence quench ratio defined as the average fluorescence of drug alone (Em1) divided by that of drug in the presence of DNA (Em2). Data represented the mean  $\pm$  SE of three independent experiments. Statistically significant differences between control and treatment groups are indicated (\*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ).

## Discussion

In an effort to develop new anti-cancer therapeutic agents, we explored the anti-tumor potential of quinoline antimalarials using MCF-7 human breast cancer cells as our model system. The results of our experiments show antimalarial compounds inhibited cell growth *in vitro*, and two mechanisms for growth inhibition were identified: (1) promotion of cell cycle exit and cell differentiation and (2) activation of p53-dependent apoptosis.

Chloroquine was the most active apoptosis-inducing agent. DNA damage is a well-established apoptotic trigger that engages p53 protein as well as downstream targets of p53 including p21(WAF1) [19, 20]. Because chloroquine intercalates into DNA [21], and stimulates both p53 and p21 (WAF1) protein expression in MCF-7 cells we hypothesize that DNA damage is involved in the apoptotic response to chloroquine. The specific mechanism by which chloroquine might create DNA damage is unclear. Chloroquine is an unusual DNA intercalator because it has a two membered planar ring structure (Figure 1); in contrast, typical DNA intercalators have three or more fused planar rings [9, 21]. In addition, the tertiary aminoalkyl side-chain of chloroquine is modeled to occupy the minor groove of DNA, and this could have consequences for many DNA binding proteins and enzymes [21, 22]. Chloroquine has been reported to inhibit mammalian topoisomerase I and II [23, 24]. Inhibition of topoisomerase II activity is a clas-



sis response to DNA intercalating agents [25, 26] and topoisomerase inhibition is a potential mechanism of action of chloroquine in MCF-7 cells. Alternatively, the 10–15-fold increase in p21(WAF1) protein elicited in MCF-7 cells by chloroquine might be sufficient to activate apoptosis in the absence of DNA damage. Sheikh et al., showed that plasmid driven p21(WAF1) over expression in human breast tumor cell lines stimulated apoptosis [27]. In either this model or the DNA damage model, induction of p21(WAF1) protein emerges as a marker that can be used to screen compounds for apoptotic activity in human breast tumor cell lines.

Quinidine also increased p53 and p21 (WAF1) protein levels and stimulated apoptosis in MCF-7 cells, but was less active than chloroquine. An interesting feature of the apoptotic response of MCF-7 cells to quinidine, was the stereoselectivity. Quinine, a stereoisomer of quinidine, did not increase p53 or p21(WAF1) protein levels and did not trigger apoptotic cell death. Quinine was also less effective than either quinidine or chloroquine in binding DNA. The stereoisomers, quinine, and quinidine should prove very valuable in elucidating the mechanisms for apoptosis by quinoline drugs.

The MCF-7 response to quinine demonstrated that apoptotic cell death was not obligatory for cell growth arrest by antimalarial agents. Chloroquine, quinidine, and quinine all increased the percentage of G0 MCF-7 cells. Cell transition into the quiescent G0 phase is prerequisite for differentiation. Using lipid droplet accumulation measured by ORO staining and induction of the MFGM as markers of mammary cell differentiation, all three quinoline antimalarials were observed to induce differentiation in MCF-7 cells. Cell differentiation therapies such as FR901228, SAHA, and pyroxamide have recently entered into clinical trials and are an active area of cancer research [28, 29]. We propose on the basis of the data presented that quinoline antimalarial drugs be considered prototype compounds for the development of novel agents to stimulate breast tumor cell differentiation. Induction of differentiation by quinine was dissociated from both p21(WAF1) and apoptosis, and we conclude that differentiation is a distinct mechanism for inhibition of cell growth by antimalarials. Based on the differential response of MCF-7 cells to quinine and quinidine, we believe that a screening system based upon Ki67 expression is preferable to p21(WAF1) for identification of compounds that promote breast tumor cell differentiation.

Previous studies in our laboratory demonstrated that differentiation of MCF-7 cells by quinidine was associated with histone H4 hyperacetylation [4]. Elucidation of the regulation of histone acetylation state by antimalarials is expected to provide important insight into how quinoline antimalarials regulate breast tumor cell differentiation.

### Acknowledgements

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Address for offprints and correspondence: JS Strobl, Department of Biochemistry and Molecular Pharmacology, Robert C Byrd Health Sciences Center, West Virginia University, Morgantown, WV, 26506, USA; Tel.: 304-293-7151; Fax: 304-293-6854; E-mail: jstrobl@hsc.wvu.edu

## UNPUBLISHED DATA

### Title Page

# PKC $\delta$ is a Pro-survival Factor in Human Breast Tumor Cell Lines

Meredith A. McCracken<sup>1</sup>, Loren J. Miraglia<sup>2</sup>, Robert A. McKay<sup>2</sup> and Jeannine S. Strobl<sup>1</sup>

Department of Biochemistry and Molecular Pharmacology and  
Genetics and Developmental Biology Program<sup>1</sup>

West Virginia University  
Morgantown, WV 26506

and

Isis Pharmaceuticals<sup>2</sup>  
Carlsbad Research Center  
Carlsbad, CA 92008

Corresponding Author:

Jeannine S. Strobl, Ph.D.

Department of Biochemistry and Molecular Pharmacology

Robert C. Byrd Health Sciences Center

PO Box 9142

West Virginia University

Morgantown, WV 26506

Phone: 304-293-7151 FAX: 304-293-6854 e-mail: [jstrobl@hsc.wvu.edu](mailto:jstrobl@hsc.wvu.edu)

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Abbreviations: IR, ionizing radiation; PKC, protein kinase C; MCF-7, Michigan Cancer Foundation; MDA-MB-231, M.D. Anderson metastatic breast; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; NF $\kappa$ B, nuclear factor  $\kappa$ B; IKK, I $\kappa$ B kinase; DMEM, Dulbecco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; FBS, fetal bovine serum; TPA, 12-O-tetradecanoylphorbol-13-acetate; ER, estrogen receptor; MDR, multi-drug resistance; ODC, ornithine decarboxylase; Ab, antibody

## Abstract

Protein kinase C (PKC) promotes cell survival in response to ionizing radiation in a variety of experimental models including human carcinoma, human glioblastoma, and transformed mouse embryo fibroblast cell lines. We have introduced specific antisense oligonucleotides into human mammary tumor cell lines *in vitro* to analyze the role of individual PKC isoforms in radiation-induced cell death in breast cancer. MDA-MB-231 and MCF-7 cells treated with oligonucleotide directed against the PKC  $\delta$  isoform exhibited impaired survival in response to 5.6Gy  $\gamma$ -radiation as measured by mitochondrial metabolism of tetrazolium dye. The role of PKC  $\delta$  in the breast tumor cell lines was of particular interest because contradictory reports exist in the literature regarding the role of PKC  $\delta$  in cell survival and apoptosis. A comparison of the effects of the PKC  $\delta$  antisense oligonucleotide and a nucleotide scrambled version of this nucleotide revealed only the antisense oligonucleotide decreased cell survival. The PKC  $\delta$  antisense oligonucleotide decreased cell survival after exposure to low (1.5Gy) radiation doses and in the absence of radiation insult. Furthermore, MCF-7 cells transformed to express a dominant negative mutant of PKC  $\delta$  exhibited reduced survival. Comet analysis showed that PKC  $\delta$  oligonucleotide treatment caused an accumulation of cells containing damaged DNA similar to that seen in 1.5Gy radiation treated cells. We conclude that PKC  $\delta$  acts as a pro-survival factor in human breast tumor cells *in vitro*.

## Introduction

The protein kinase C (PKC) family of serine/threonine kinases mediate intracellular responses to a variety of stimuli, including growth factors, hormones and neurotransmitters. PKC is widely distributed in mammalian tissues. Some isoforms are expressed ubiquitously while the expression of other isoforms is restricted to specific tissues. Twelve PKC isoforms are distinguished on the basis of protein homology and cofactor utilization. PKC isoforms are divided into three subfamilies: classical ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\lambda$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ , and  $\mu$ ) and atypical ( $\zeta$  and  $\iota/\gamma$ ). Classical PKC isoforms possess a calcium binding domain and two cysteine-rich zinc fingers that are involved in diacylglycerol (DAG) binding. While the novel PKC isoforms contain the DAG binding sites, they lack the calcium binding domain and differ from the atypical isoforms, which require neither calcium nor DAG for activation. The PKC isoform profile determined in the human mammary epithelial cell line, MCF-7, includes PKC  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\gamma$ ,  $\iota$ ,  $\mu$  and  $\zeta$  isoforms. MDA-MB-231 human mammary epithelial cell lines express a PKC isoform profile very similar to MCF-7 cells with the exception that PKC  $\alpha$  is highly expressed in MDA-MB-231 cells and weakly expressed in MCF-7 cells (Morse-Gaudio et al., 1998; Ways et al., 1995).

PKC participates in abnormal growth processes such as carcinogenesis, tumor progression, and inflammation (Goekjian and Jirousek, 1999). As the primary intracellular receptor for the tumor promoting phorbol esters, PKC plays a role in stimulating early events in tumor formation. Proliferative signals converging on mitogen-activated protein kinase (MAPK) from G-protein coupled receptors and the receptor tyrosine kinases for epidermal- and platelet-derived growth factors are transduced through PKC (van Biesen et al., 1995) and could participate in tumor promotion. In addition, a role for PKC in the later stages of tumor development is suggested by

the observation that the metastatic capacity of tumor cells and PKC activity are positively correlated (Carey et al., 1999). Activation of PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) has been shown to increase extracellular matrix attachments, while reduction of these attachments has been observed in response to PKC inhibition (Herbert, 1993). Overexpression of the PKC  $\alpha$  isoform in MCF-7 cells resulted in reduced extracellular matrix attachments by alterations in integrin heterodimer expression and facilitated metastatic growth (Carey et al., 1999).

PKC overexpression and increased PKC activity have been observed in human breast cancers compared with normal mammary tissue (O'Brian et al., 1989; Gorge et al., 1996). Furthermore, there is a correlation between elevated PKC protein levels and aggressive breast cancer phenotypes, such as those that lack estrogen receptors (ER) and exhibit multidrug resistance (MDR) (Borner et al., 1987; Lee et al., 1992). Relatively little is known about cell type specific functions of the individual isoforms in mammary epithelium, however overexpression of PKC  $\alpha$  and PKC  $\delta$  in mammary cells elicited responses that were clearly distinct from those in some other cell types. Overexpression of PKC  $\alpha$ , stimulated MCF-7 breast tumor and C6 glioma cell growth (Ways et al., 1995; Baltuch et al., 1995), but overexpression of this same PKC isoform inhibited growth of bovine aortic endothelial cells and rat embryonic smooth muscle cells (Rosales et al., 1998; Wang et al., 1997). Additionally, PKC  $\delta$  overexpression in rat mammary adenocarcinoma cells stimulated anchorage independent growth (Kiley et al., 1999a), but inhibited growth of cells of ovarian origin (CHO) as well as of mouse NIH3T3 cell fibroblasts (Watanabe et al., 1992; Mischak et al., 1993). A possible mechanism for inhibition of growth by PKC  $\delta$  is through its reported function as a caspase 3 substrate during apoptosis (Cross et al., 2000). These observations indicate that generalizations about the role of particular PKC isoforms

without regard to cell context may not be valid. We conclude that cellular origins and the PKC isoform profiles are both important considerations in assigning cell type specific functional roles of PKC.

We have applied antisense oligonucleotide technology to explore isoform specific functions of PKC in the survival of human mammary carcinoma cell lines *in vitro*. We demonstrate that oligonucleotides can be used to selectively eliminate PKC  $\delta$  from these cells and provide evidence that PKC  $\delta$  functions as a pro-survival factor in human breast tumor cell lines. Further, our findings suggest that down-regulation of PKC  $\delta$  may be a useful approach to growth inhibition in mammary tumors.

## MATERIALS AND METHODS

### *Cell Culture*

MDA-MB-231 and MCF-7 (passage #39-50) human mammary tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Summit Biotechnology, Fort Collins, CO) and 0.04 mg/ml gentamicin in a 7.5% CO<sub>2</sub>, 37°C, humidified incubator. Cells were passaged weekly at 1:10 or 1:5 ratios, respectively. MCF10A (passage #1-20) immortalized human mammary epithelial cells (American Type Culture Collection, Manassas, VA) were maintained in Mammary Epithelial Cell Growth Medium (MEGM) (Bio Whittaker) in a 7.5% CO<sub>2</sub>, 37°C, humidified incubator and passaged weekly at a 1:4 ratio.

### *Antisense Oligonucleotides*

MDA-MB-231 (1.1x10<sup>5</sup>/35 mm<sup>2</sup> dish), MCF-7 (2.2x10<sup>5</sup>/35 mm<sup>2</sup> dish) or MCF10A (1.5x10<sup>5</sup>/35 mm<sup>2</sup> dish) cells were treated for 4-5 h with 100-200 nM PKC  $\delta$  methoxy-ethoxy modified antisense oligonucleotide (ISIS #13513) or the respective nucleotide scrambled version of the oligonucleotide (ISIS #13514) (Isis Pharmaceuticals, Carlsbad, CA) in 3  $\mu$ l Lipofectin (Life Technologies, Rockville, MD) transfection reagent/1 mL Opti-Mem I reduced serum medium (Life Technologies). Transfection was stopped by medium exchange with DMEM/2% FBS or MEGM (MCF10A).

### *Radiation*

Cells were exposed to 1.5-5.6 Gy doses of  $\gamma$ -ionizing radiation delivered with a Cesium<sup>137</sup> source in a Gammacell 40 (Atomic Energy of Canada Ltd., Ottawa) at 108.7 rads/min or a



Gammacell 1000 (Atomic Energy of Canada Ltd.) at 730.0 rads/min under ambient temperature and atmospheric conditions. The clonogenic survival curves generated at these two radiation rates were similar. After radiation medium was replaced with DMEM/10%FBS.

*MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay*

Metabolism of MTS was used as index of cell viability. MDA-MB-231, MCF-7 or MCF10A cells were plated at 250 or 2500 (MCF-7) cells/well in a 96-well plate in 225  $\mu$ l DMEM/10% FBS or MEGM. After plating (5 or 7 (MCF-7) days), fresh growth medium (100  $\mu$ l DMEM/5% FBS or MEGM + 20  $\mu$ l Cell Titer 96) (Promega, Madison, WI)) was added. Conversion of MTS reagent to formazan product was measured after a 2 h incubation at 37°C by an increase in absorbance at 490 nm using a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The MTS assay was linear under our assay conditions for 3 h.

*Western Blotting*

MDA-MB-231 ( $4.5 \times 10^5$ /100 mm<sup>2</sup> dish), MCF-7 ( $7.0 \times 10^5$ /100 mm<sup>2</sup> dish) or MCF10A ( $1.5 \times 10^6$ /100 mm<sup>2</sup> dish) cells were rinsed one time with PBS. Total cellular proteins were collected by syringe in 100-150  $\mu$ l boiling lysis buffer (1% SDS, 10mM Tris, pH 7.4) and chilled on ice. Proteins were boiled for 5 min then centrifuged at 4°C, 13,000 rpm in an Eppendorf centrifuge 5415 C (Brinkmann Instruments Inc., Westbury, NY). Aprotinin (1 mM), leupeptin (1 mM) and phenylmethyl sulfonyl fluoride (100  $\mu$ M) were added to the supernatant. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Protein samples were diluted in 1X sample buffer (0.06 M Tris-HCl, 10% glycerol, 2% SDS, 0.2% bromphenol

blue, 2.5% 2-mercaptoethanol, pH 6.8)/1 mM dithiothreitol and then resolved on 7.5% acrylamide gels at 100 V. Proteins were transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA) at 25 V for 2 h. Purified PKC  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\zeta$  proteins (#539674, #539673, Calbiochem, San Diego, CA) were used as standards. Membranes were blocked at 4°C, overnight in 3% non-fat dry milk/Tris buffered saline + 0.05% Tween-20. Membranes were incubated with PKC  $\alpha$ ,  $\delta$  or  $\epsilon$  mouse monoclonal antibody (#P16520, #P36520, #P14820, BD Transduction Laboratories, San Diego, CA), PKC  $\zeta$  goat polyclonal Ab, PKC  $\eta$  rabbit polyclonal Ab or bcl-2 mouse monoclonal Ab (SC #216-G, #215, #509, Santa Cruz, CA) for 3 h 15 min (1:250 dilution for all primary Abs). Membranes were hybridized with a 1:1000 dilution of anti-mouse (SC #2005), 1:8000 of anti-rabbit (SC #2004) or a 1:10,000 dilution of anti-goat (SC #2020) horseradish peroxidase-conjugated secondary antibody for 30 min. Signals were visualized by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to film. Signals were quantitated by FluorChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction and normalized to a 50 or 200 kDa protein band on the Gel Code Blue (Pierce) stained acrylamide gel.

### *Comet Analysis*

Cells were treated with PKC  $\delta$  oligonucleotides for 24-48 h. Cells were harvested and resuspended at  $1.5 \times 10^5$  cells/ml in ice cold PBS. Cells were maintained on ice and treated with 1.5 Gy radiation. Following radiation, 50  $\mu$ l of PBS cell suspension was mixed with 500  $\mu$ l of 42°C low melting point agarose. Seventy-five  $\mu$ l of cell suspension were spread evenly onto a Comet Slide (#4250-050-03 Trevigen, Gaithersburg, MD) and allowed to dry flat in the refrigerator for 30 min. Slides were then immersed in prechilled lysis solution (#4250-050-01,

Trevigen) and incubated on ice for 45 min, then transferred to freshly prepared alkali solution (300 mM NaOH, 1 mM EDTA-pH 8.0) for 45 min at room temperature, protected from light. Slides aligned equidistant from the electrodes were electrophoresed for 30 min at 1 V/cm and 300 mA. Slides were immersed in 70% ethanol for 5 min and then allowed to air dry overnight at room temperature. Slides were stained with 50  $\mu$ l of SYBR Green stain [1000X] for 7 min. The comets were visualized using a Nikon Eclipse TS100 microscope with 63X objective and FITC-filter cube. Comet images were captured and analyzed using the LAI Automated Comet Assay Analysis System (Loats Associates, Inc. Westminster, MD). The tail moment [(%DNA)(distance traveled)] was used for quantitative analysis of DNA damage for 80 comets per treatment/experiment.

### *Plasmids*

PKC  $\delta$  dominant negative plasmid constructs were generously provided by Dr. Bernard Weinstein (Herbert Irving Comprehensive Cancer Center, Columbia University). MCF-7 cells ( $1 \times 10^6$ /100 mm<sup>2</sup> dish) were transformed with 10  $\mu$ g of the empty vector or 13.7  $\mu$ g of PKC  $\delta$  dominant negative (normalized for the neomycin resistance gene copy number) by the DOTAP transfection reagent (#1811177, Boehringer Mannheim, Germany). In accordance with the manufacturers protocol, 530  $\mu$ l of a 1:4 dilution of DOTAP reagent: 20 mM HEPES (#391333, Calbiochem) buffer (pH 7.4) were combined with HEPES buffer containing 0.1  $\mu$ g/ml of plasmid DNA. The DNA/DOTAP mixture was incubated at room temperature for 15 min and then added directly to culture medium (9.5 ml DMEM/2% FBS). After 48 hours, medium was exchanged with 10 ml of DMEM/5% FBS. On the following day, medium was exchanged with 10 ml DMEM/10% FBS + 400  $\mu$ g/ml of G418 (#20039, Stratagene, La Jolla, CA). Following 11

days growth in selection medium, trypan blue excluding viable cells were counted on a hemocytometer or whole cell protein extracts were prepared.

### *Statistical Analysis*

Statistically significant differences ( $P < 0.05$ ) were determined using Student's t-test. For multiple comparisons one-way ANOVA with Tukey's multiple comparison test was used.

## RESULTS

### *Differential expression of PKC isoforms in MDA-MB-231 and MCF-7 cells*

Individual PKC isoforms are differentially expressed within human mammary tumor cells. Western blot analysis of MDA-MB-231 and MCF-7 cell extracts was performed to examine the relative protein levels of PKC  $\alpha$ , PKC  $\delta$ , PKC  $\epsilon$ , PKC  $\eta$  and PKC  $\zeta$  (Fig. 1). The major difference in the PKC expression profile between these two cell lines is the PKC  $\alpha$  isoform expression. PKC  $\alpha$  is present at higher levels in MDA-MB-231 cells than in MCF-7. MDA-MB-231 and MCF-7 cells also differed in their expression of the PKC  $\eta$  isoform. While, MCF-7 cells contain two species of PKC  $\eta$ , a faster and slower migrating form, PKC  $\eta$  was undetectable in MDA-MB-231 cells. The expression of PKC  $\delta$ , PKC  $\epsilon$  and PKC  $\zeta$  were similar between the two cell lines, with slightly stronger signals detected in MCF-7 cell extracts.

### *PKC $\delta$ oligonucleotide decreases survival in human breast tumor cells*

PKC gene and protein expression are induced following ionizing radiation (Woloschak et al., 1990; Kim et al., 1992). PKC inhibition radiosensitizes human squamous carcinoma, human colon adenocarcinoma, transformed mouse embryo fibroblasts and human glioblastoma cell lines (Hallahan et al., 1992; Zaugg et al., 2001; Rocha et al., 2000; Begemann et al., 1998). Our goal was to examine the roles of specific PKC isoforms in response to radiation insult by 5.6 Gy on cell survival using mitochondrial metabolism of MTS as an end-point. This assay is rapid, reproducible and suitable for screening purposes. Using the MTS assay, the human mammary tumor cell lines, MDA-MB-231 and MCF-7, used for these studies showed similar survival curves in response to 0.75-9.5 Gy radiation (data not shown). Oligonucleotide that targeted the novel PKC  $\delta$  isoform was introduced into the two human breast tumor cell lines using liposomes.

Treatment of MDA-MB-231 cells with PKC  $\delta$  oligonucleotide decreased cell survival ( $P < 0.05$ ) in the irradiated cells by 44% compared with cells that were treated with the nucleotide scrambled version of this oligonucleotide (Fig. 2A). Liposome treatment alone or liposome treatment plus the nucleotide scrambled version of the PKC  $\delta$  oligonucleotide had no effect on MDA-MB-231 survival indicating that the oligonucleotide sequence was critical to the decreased survival. We conclude that the influence of PKC upon breast tumor cell survival following ionizing radiation and is an isoform specific effect, and that PKC  $\delta$  oligonucleotide effects on the survival of irradiated MDA-MB-231 cells are quite specific.

In MCF-7 cells (Fig. 2B), liposome treatment alone reduced survival of irradiated cells by approximately 50%, and the magnitude of the liposome effect exceeded that of oligonucleotide treatment in affecting cell survival after gamma irradiation. Nevertheless, PKC  $\delta$  oligonucleotide significantly decreased the survival of MCF-7 cells after radiation treatment, thus providing confirmatory evidence for the importance of this PKC isoform in the radiation survival of human breast tumor cell lines.

#### *PKC $\delta$ oligonucleotide specifically reduced PKC $\delta$ protein levels*

To further analyze the role of PKC  $\delta$  oligonucleotide in breast tumor cell survival post-irradiation, we tested whether PKC  $\delta$  protein levels were decreased in cells treated with oligonucleotide that targeted PKC  $\delta$ . Whole cell extracts were prepared from MCF-7 and MDA-MB-231 cells either 52 h or 72 h after transfection with PKC  $\delta$  oligonucleotide. No PKC  $\delta$  protein was detected in either cell line after 72 h (Fig. 3). Untreated cells, cells treated with lipofectin alone or the inactive nucleotide scrambled version of the PKC  $\delta$  oligonucleotide showed constant levels of PKC  $\delta$  at 52 h and 72 h. We conclude that the PKC  $\delta$  oligonucleotide

transfection protocol, but not the control conditions used in the radiation survival experiments, depleted human breast tumor cell lines of PKC  $\delta$  protein. To test whether the effect of PKC  $\delta$  oligonucleotide was selective for the  $\delta$  isoform of protein kinase C, protein levels of PKC  $\epsilon$  and PKC  $\zeta$  were assayed in the MCF-7 cell extracts (Fig. 3). There was no effect of the PKC  $\delta$  oligonucleotide on levels of either PKC  $\epsilon$  or PKC  $\zeta$ .

Bcl-2 is an anti-apoptotic protein that is highly expressed in human breast tumor cells (Eissa et al., 1999). We tested whether the effect of PKC  $\delta$  oligonucleotide could be manifested as a decrease in bcl-2 protein levels by immunoblot analysis. Figure 3 shows that the levels of bcl-2 protein are constant in MCF-7 and MDA-MB-231 cells treated with the PKC  $\delta$  oligonucleotide. The decreased survival of irradiated cells following depletion of PKC  $\delta$  protein with PKC  $\delta$  oligonucleotide can not be attributed to a decrease in bcl-2 protein. We hypothesize that PKC  $\delta$  protein is a survival factor in these two human breast tumor cell lines, and that oligonucleotide depletion of PKC  $\delta$  decreases cell survival in response to  $\gamma$ -radiation.

#### *PKC $\delta$ oligonucleotide decreased breast tumor cell survival in response to 1.5 Gy $\gamma$ -radiation*

Breast cancer radiation therapy is delivered in the range of 1-2 Gy/treatment. To determine whether PKC  $\delta$  oligonucleotide would decrease breast tumor cell survival *in vitro* in this radiation dose range, cells were exposed to 1.5 Gy radiation 17 h after transfection with oligonucleotides (Fig. 4). Control, non-irradiated cells were treated in parallel with PKC  $\delta$  oligonucleotide or its nucleotide scrambled version. When MCF-7 cells were pre-treated with the PKC  $\delta$  oligonucleotide, there was no significant difference in the cell or clonogenic survival in comparison to treatment with the scrambled oligonucleotide (data not shown). However, PKC  $\delta$  oligonucleotide treatment impaired MDA-MB-231 cell survival at the 1.5 Gy radiation dose.

Consistent with earlier results (Fig. 2A), a 40% decrease in cell survival over 5 days was observed in non-irradiated MDA-MB-231 cells treated with the PKC  $\delta$  compared with the nucleotide scrambled oligonucleotide (Fig. 4). Survival of the MDA-MB-231 cells exposed to the low dose of  $\gamma$ -radiation (1.5 Gy) alone was decreased approximately 20% compared to non-irradiated controls. Pre-treatment of the cells with the PKC  $\delta$  oligonucleotide caused cell survival to decrease by approximately 70%. Cell survival in irradiated cells pre-treated with the PKC  $\delta$  oligonucleotide was significantly reduced ( $P < 0.05$ ) compared with the cells that were pre-treated with the nucleotide scrambled version of the oligonucleotide. We conclude that PKC  $\delta$  oligonucleotide is a survival inhibitor in MDA-MB-231 human breast tumor cells.

#### *Transformation of MCF-7 cells with PKC $\delta$ dominant negatives*

Plasmid vectors encoding a dominant negative form of PKC  $\delta$  (Soh et al., 1999) were introduced into MCF-7 cells. The presence of G418 in cell growth medium permitted selection for MCF-7 cells with stable expression of the neomycin resistance gene within the plasmid vector. MCF-7 cells that expressed a dominant negative PKC  $\delta$  mutant demonstrated approximately 60% fewer viable cells than cells transformed with the empty vector alone (Fig. 5A). Western blot analysis was used to examine the total PKC  $\delta$  protein levels in extracts prepared from empty vector or PKC  $\delta$  dominant negative transformed MCF-7 cells. This approach was utilized to confirm PKC  $\delta$  dominant negative expression. The PKC  $\delta$  antibody used in these experiments recognized the cysteine rich repeat domain in amino terminal portion of PKC  $\delta$ . The dominant negative mutation within the carboxy terminal ATP binding domain of PKC  $\delta$  should not interfere with antibody recognition. MCF-7 cells transformed with PKC  $\delta$  that contained a dominant negative point mutation expressed 3.5-fold ( $n=3$ ,  $P < 0.05$ ) higher levels of



immunodetectable PKC  $\delta$  consistent with expression of vector derived PKC  $\delta$  (Fig. 5B). Perturbation of endogenous PKC  $\delta$  had no effect on bcl-2 protein levels. These findings indicate that inhibition of PKC  $\delta$  impairs cell growth and confirm that PKC  $\delta$  is a survival factor in breast tumor cells.

#### *PKC $\delta$ oligonucleotide treatment resulted in loss of DNA integrity*

Single cell gel electrophoresis or “comet analysis” performed under alkali conditions permits sensitive and quantitative analysis of single- and double-strand DNA damage (Fairbairn et al., 1995). The electrophoretic mobility and relative intensity of fragmented nuclear DNA are incorporated into the tail moment parameter. The frequency of comets in control MDA-MB-231 was highest for tail moments less than 2, representative of cells with undamaged nuclear DNA (Fig. 6). Treatment of MDA-MB-231 cells with PKC  $\delta$  oligonucleotide caused a shift in the comet distribution toward increased tail moments, indicative of damaged DNA, while the scrambled nucleotide control did not. Treatment with 1.5 Gy  $\gamma$ -radiation caused an increase in the frequency of comets with tail moments greater than 2, similar to the effect of PKC  $\delta$  oligonucleotide treatment. Radiation induced DNA damage was undetectable when radiation treatments were performed at room temperature followed by an additional 30 minutes incubation at 37°C consistent with rapid repair of radiation induced DNA damage (data not shown). The combination of PKC  $\delta$  oligonucleotide and radiation produced an increase in the number of DNA strand breaks. Table I summarizes the comet analysis results presented in Figure 6 for analysis performed at 24 h after treatment of MDA-MB-231 cells with PKC  $\delta$  oligonucleotide. Identical experiments performed with analysis at 48 h showed that damage produced by PKC  $\delta$  oligonucleotide or PKC  $\delta$  oligonucleotide plus radiation treatments was persistent (PKC  $\delta$

TM>2=54.6%, >20=23.3%; PKC $\delta$  +IR TM>2= 70.0%, >20= 28.3%). PKC  $\delta$  antisense oligonucleotide caused unrepairable DNA damage that persisted for 48 h. We conclude that depletion of PKC  $\delta$  from MDA-MB-231 cells by antisense oligonucleotides results in a disruption of nuclear DNA.

## DISCUSSION

In the present study, we showed that selective depletion of the PKC  $\delta$  isoform decreased human mammary tumor cell survival with a concomitant increase in cells containing damaged DNA. Our findings suggest that PKC  $\delta$  positively regulates survival in breast cancer cells. Gamma radiation was used to challenge survival of the breast tumor cells in these studies because the results of earlier experiments with the PKC inhibitors, staurosporine, sangivamycin and H7 provided evidence that PKC activation was radioprotective (Begemann et al., 1998; Hallahan et al., 1992; Zhang et al., 1993). PKC activation opposes the pro-apoptotic effects of radiation-induced ceramide generation (Haimovitz-Friedman et al., 1994), but the mechanism(s) for this response and the roles of the individual PKC isoforms are not well defined. This report begins to clarify the role of individual PKC isoforms that mediate survival in breast tumor cells in response to gamma radiation.

The two main approaches to isoform selective PKC inhibition are site selective enzyme inhibition or suppression of the mRNA levels. Oligonucleotides that target RNA are highly specific PKC isoform antagonists, and may be easier and less expensive to synthesize than traditional small molecule inhibitors targeted to the enzyme (Glazer, 1998). A few small molecule inhibitors offer PKC isoform specific inhibition. The small molecule inhibitor, rottlerin, is selective for the PKC  $\delta$  isoform ( $IC_{50}$  3-6 $\mu$ M) but also inhibits calmodulin kinase III ( $IC_{50}$ =5.3 $\mu$ M) (Way et al., 2000). As an alternative to these methods, expression of PKC  $\delta$  containing a point mutation in the ATP binding domain was shown to have dominant negative activity (Soh et al., 1999). Our approach to elucidate the role of particular PKC isoforms involved in mammary tumor cell survival following radiation has been to use antisense

oligonucleotides, dominant negative protein species and where possible, small molecule inhibitors.

PKC  $\delta$  is a novel PKC. This calcium independent isoform has been proposed to both regulate and serve as a substrate for caspases (Basu and Akkaraju, 1999). Specifically, PKC  $\delta$  is activated by caspase 3 during apoptosis in leukemia cells resulting in phosphorylation of lamin B (Cross et al., 2000), an event known to precede nuclear lamina disassembly during apoptosis. In response to TPA, relocalization of cytoplasmic PKC  $\delta$  to the mitochondria of MCF-7 cells, preceding cytochrome c release has been observed (Majumder et al., 2000). These findings support a pro-apoptotic role for PKC  $\delta$ .

In contrast, growth promoting activities of PKC  $\delta$  have also been observed in mammary tumor cells. PKC  $\delta$  involvement in cytoskeleton-dependent processes is evident in MTLn3 mammary tumor cells. Expression of the inhibitory PKC  $\delta$  regulatory domain (RD  $\delta$ ) inhibited growth in soft agar, cell motility and attachment (Kiley et al., 1999b). Furthermore, in highly metastatic mammary tumor cells, PKC  $\delta$  protein and mRNA are significantly increased relative to the less metastatic tumor cells (Kiley et al., 1999a; Kiley et al., 1996).

Ornithine decarboxylase (ODC) is an enzyme essential to cell proliferation. ODC is capable of inducing cell transformation when overexpressed and is frequently highly expressed in tumor cells. Dominant negative PKC  $\delta$  expression in murine papilloma cells attenuates induction of ODC in response to oxidative damage (Otieno and Kensler, 2000). Thus, PKC  $\delta$  might act to promote cell growth by facilitating the expression of ODC.

PKC  $\alpha$  is a molecular target which shows promise clinically for development of new anti-cancer therapeutic agents. The calcium dependent PKC  $\alpha$  isoform has been shown to be involved with the acquisition of multi-drug resistant (Yu et al., 1991) and ER negative breast cancer cell

phenotypes. Stable expression of PKC  $\alpha$  in MCF-7 cells results in increased proliferation and anchorage-independent growth, as well as in a reduction of ER expression and estrogen mediated gene expression (Ways et al., 1995). Inhibition of PKC  $\alpha$  by ISIS 3521 antisense oligonucleotide has shown anti-tumor activity in phase I and II clinical trials in solid tumors (Nemunaitis et al., 1999). ISIS 3521 is now undergoing phase III clinical trials. Our results identify PKC  $\delta$  as a potential new molecular target for breast cancer drug development and support further investigation of ISIS 13513 as a therapeutic agent.

## **Acknowledgements**

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<b>Tail Moment:</b>	<b>24h</b>	
	<b>&gt;2,</b>	<b>&gt;20</b>
<b>Control</b>	<b>39.6%,</b>	<b>2.5%</b>
<b>scr</b>	<b>32.5%,</b>	<b>5.8%</b>
<b>PKC<math>\delta</math></b>	<b>60.4%,</b>	<b>16.3%</b>
<b>IR</b>	<b>60.4%,</b>	<b>17.9%</b>
<b>scr+IR</b>	<b>59.2%,</b>	<b>17.1%</b>
<b>PKC<math>\delta</math>+IR</b>	<b>70.4%,</b>	<b>27.1%</b>

Table 1

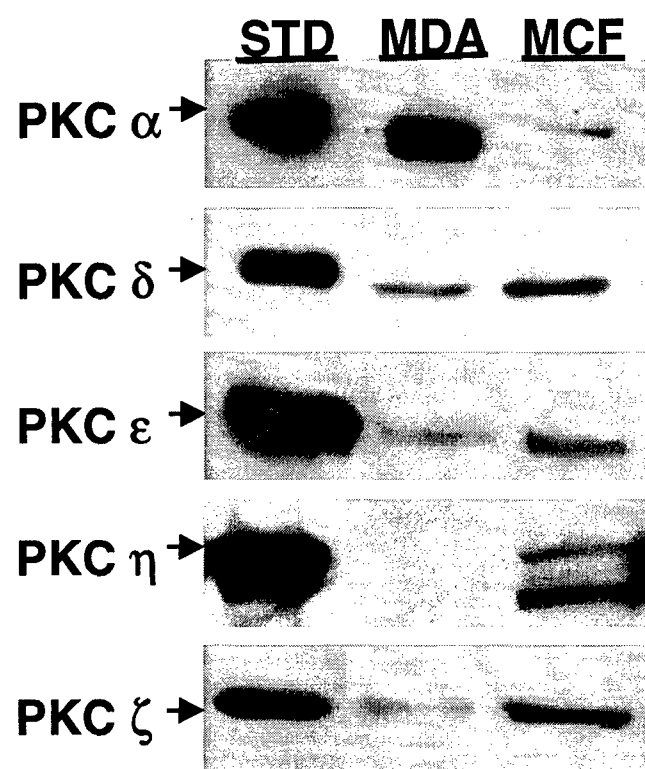
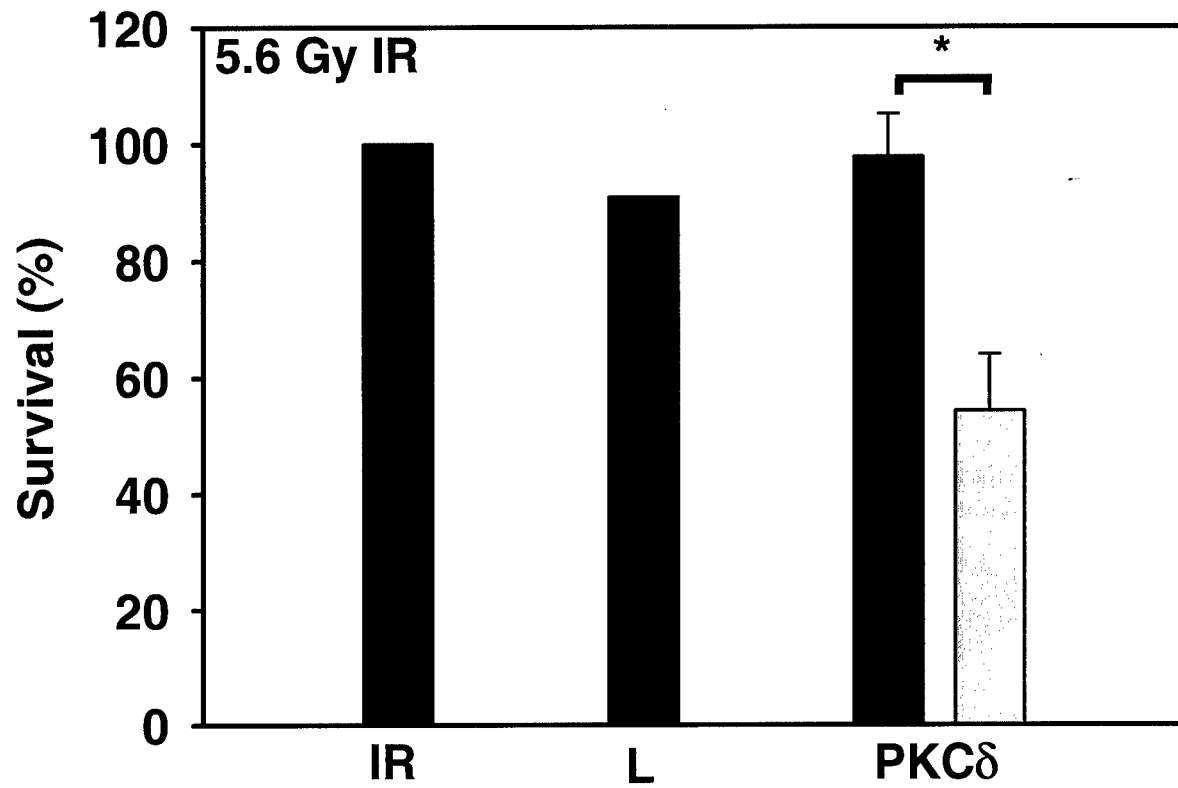


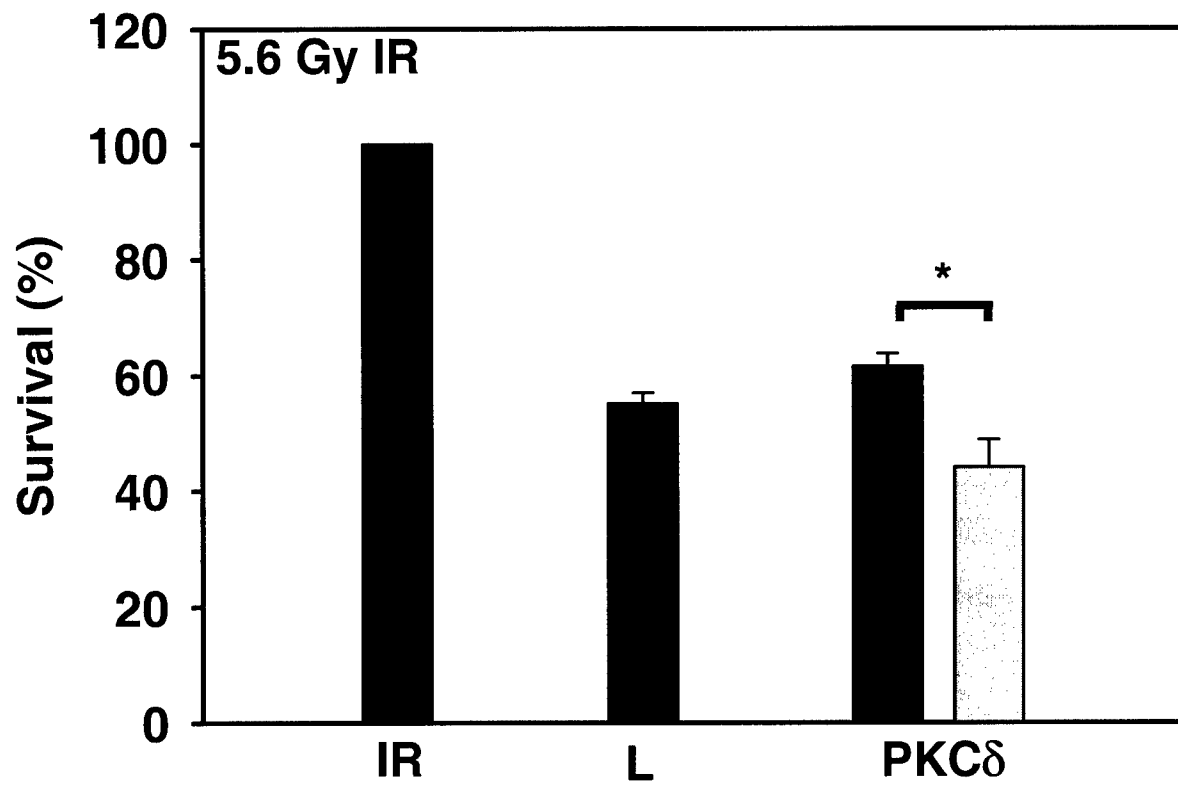
Figure 1

Figure 2

(A)



(B)





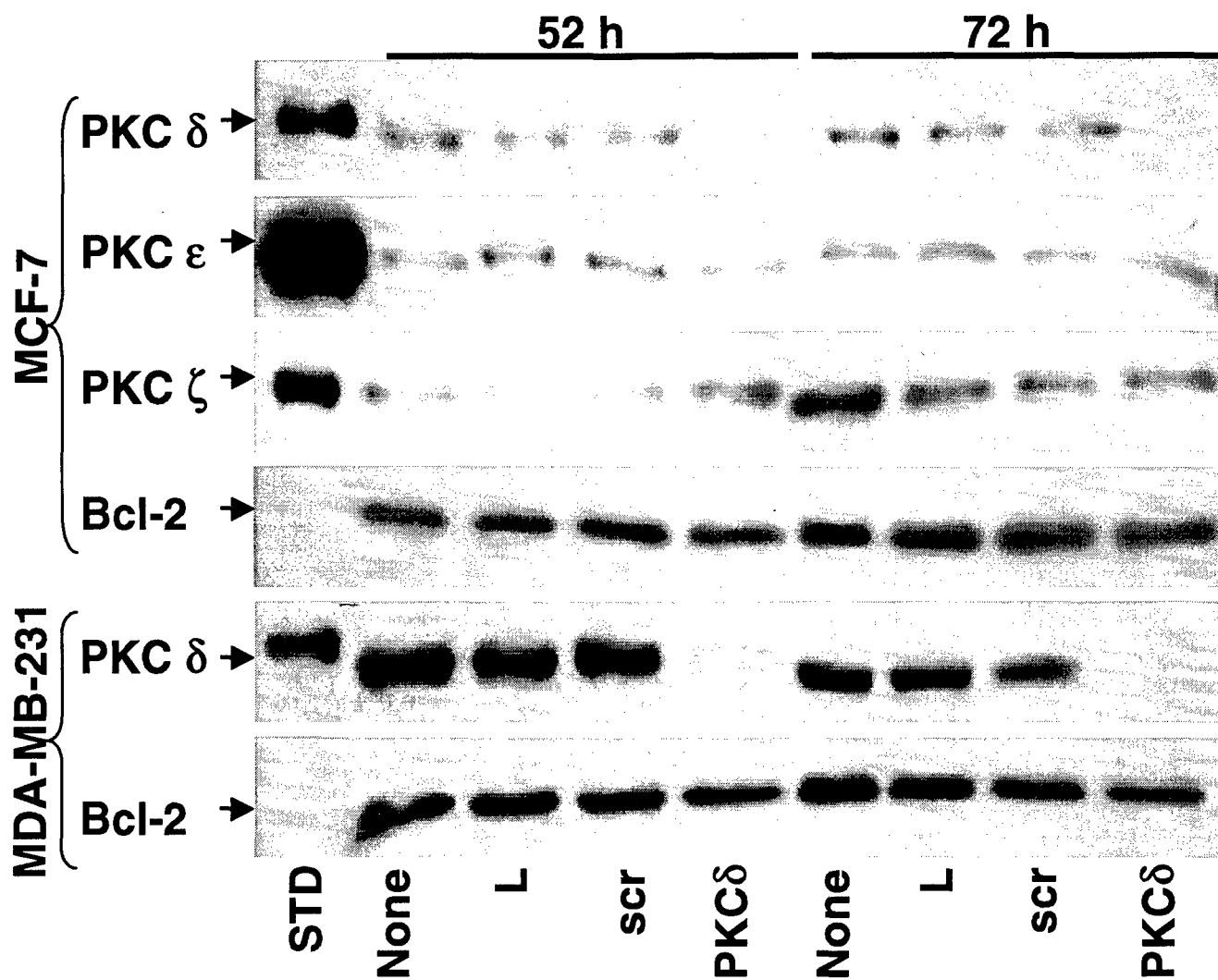


Figure 3

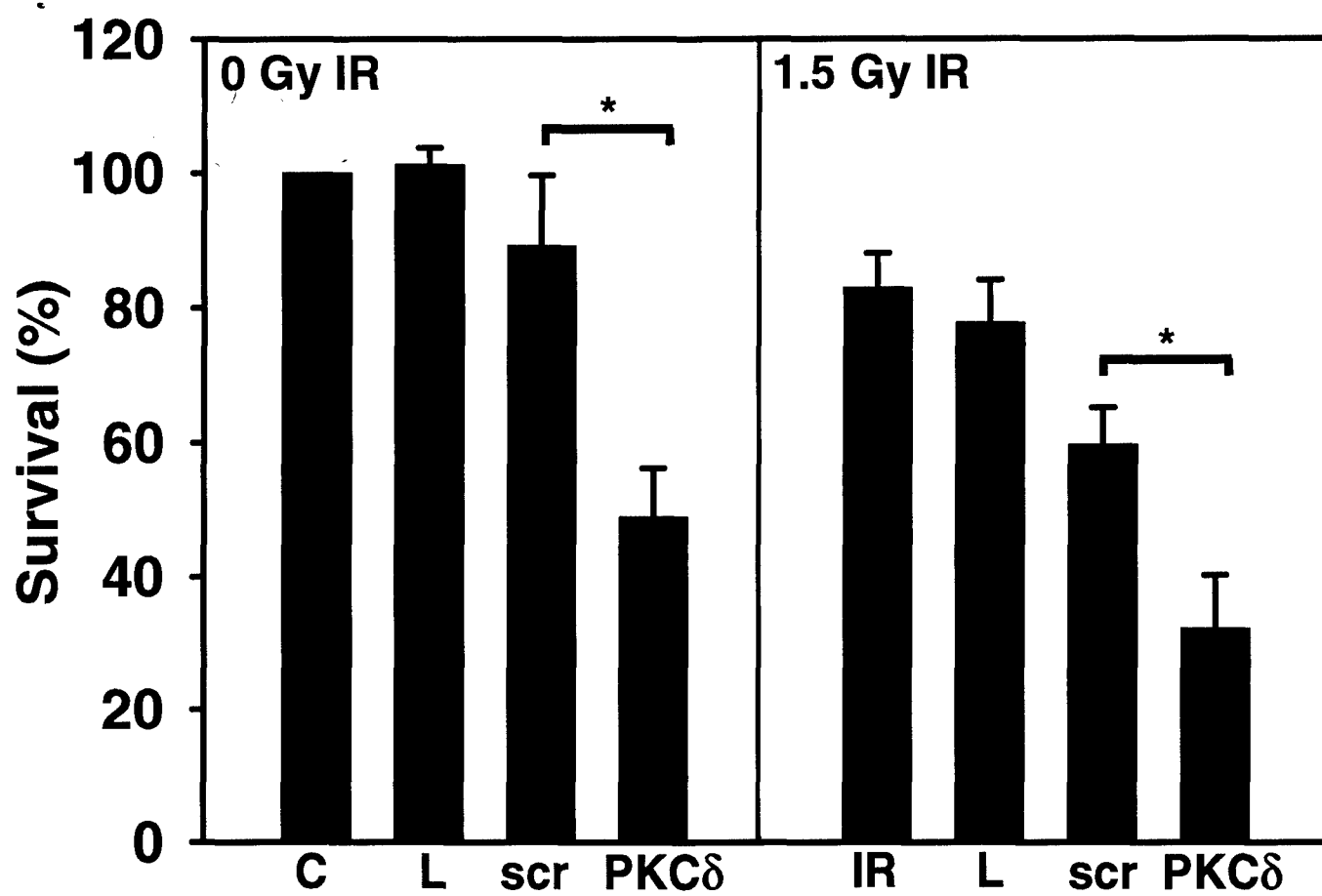
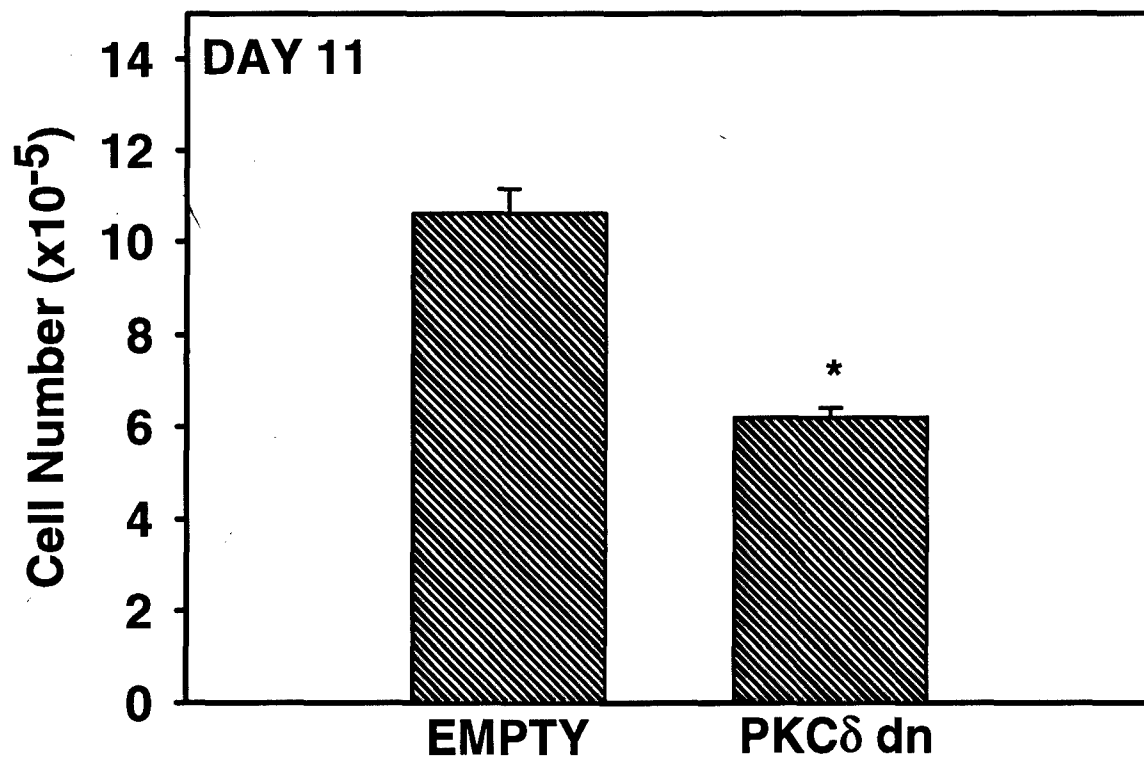


Figure 4

Figure 5

(A)



(B)

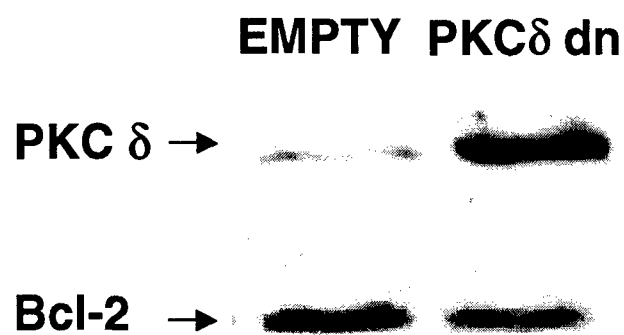
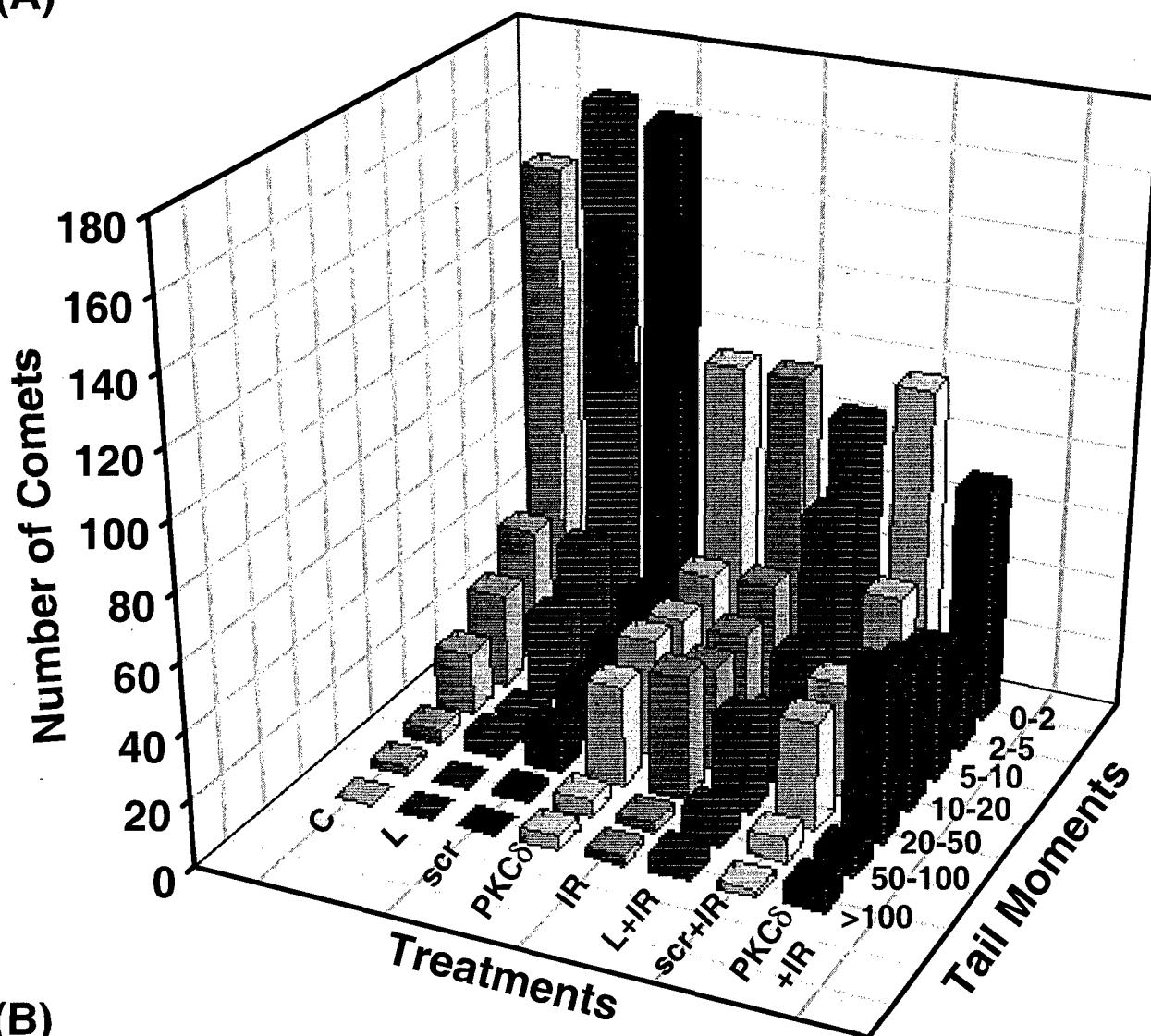


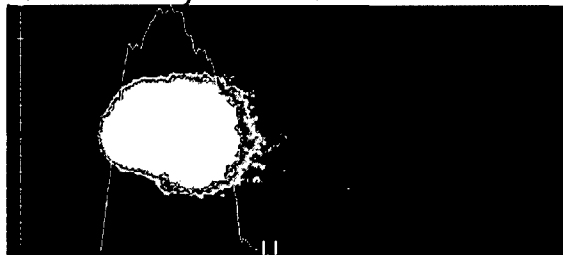
Figure 6

(A)



(B)

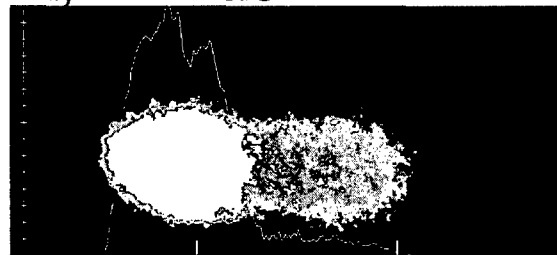
Control, TM=0.0



scr, TM=0.2



IR, TM=14.8



PKC $\delta$ , TM=18.0



**Table 1-** Comet analysis summary for PKC  $\delta$  oligonucleotide treatment of MDA-MB-231 cells.

Data are summarized from Figure 6 and expressed as the percentage of comets with tail moments  $>2$  and  $>20$  for each treatment group.

**Figure 1-** PKC protein levels in MDA-MB-231 and MCF-7 cells. Extracts were prepared from MDA-MB-231 ( $1 \times 10^6/100 \text{ mm}^2$  dish) or MCF-7 ( $7.5 \times 10^5/100 \text{ mm}^2$  dish) cells 24 h post-subculture from confluency. Proteins ( $40 \mu\text{g/lane}$ -  $\alpha$ ,  $\epsilon$ ,  $\eta$ ,  $\zeta$  and  $62 \mu\text{g/lane}$ -  $\delta$ ) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC  $\alpha$ , PKC  $\delta$ , PKC  $\epsilon$ , PKC  $\eta$  and PKC  $\zeta$ . The PKC standards (STD) included purified PKC  $\alpha$  (15 ng/lane), PKC  $\delta$  (15 ng/lane), PKC  $\epsilon$  (5 ng/lane), PKC  $\eta$  (3 ng/lane) and PKC  $\zeta$  (40 ng/lane). Data shown are typical of  $n=2$  experiments.

**Figure 2-** Cell survival in response to  $\gamma$ -radiation +/- PKC  $\delta$  oligonucleotide treatment. A, MDA-MD-231 cells were treated with 100 nM oligonucleotide plus liposome that targets PKC  $\delta$  (gray bar). Controls were treated with radiation alone (IR), radiation plus liposome (L), or radiation plus liposome plus scrambled nucleotide versions of this oligonucleotide (black bars). Post-transfection (24 h), cells were irradiated with 5.6 Gy of  $\gamma$ -irradiation, harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. B, MCF-7 cells were treated with 200 nM oligonucleotide that targets PKC  $\delta$  in an isoform-specific manner. Cells were irradiated with 5.6 Gy  $\gamma$ -radiation 48 h post-transfection, harvested and replated in 96-well plates at a cloning density of 2500 cells/well. After 7 days of incubation, cell survival was estimated using the MTS assay. Data are the mean  $\pm$  SE of  $n=4$  (A) or  $n=3$  (B) independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. Statistically significant differences between treatment groups receiving PKC  $\delta$  oligonucleotide versus nucleotide scrambled sequence PKC oligonucleotide are indicated (\* $P<0.05$ ).

**Figure 3-** Immunoblot analysis of PKC  $\delta$  oligonucleotide treated cells. A, Extracts were prepared from cells 52-72 h after treatment with lipofectin alone (L), PKC  $\delta$  oligonucleotide (PKC  $\delta$ ), the nucleotide scrambled version (scr) or no treatment. Proteins (20  $\mu$ g/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC  $\delta$ , PKC  $\epsilon$ , PKC  $\zeta$  and bcl-2. The PKC standards (STD) included purified PKC  $\delta$  (15 ng/lane), PKC  $\epsilon$  (5 ng/lane) and PKC  $\zeta$  (25 ng/lane). Results are typical of three independent experiments.

**Figure 4-** PKC  $\delta$  oligonucleotide impaired human breast tumor cell survival in response to low dose  $\gamma$ -radiation. MDA-MB-231 cells were transfected with PKC  $\delta$  oligonucleotides and 17 h later irradiated with 1.5 Gy  $\gamma$ -radiation, harvested and replated (250 cells/well) in 96-well plates. MTS activity was measured after 5 days as an index of survival. Data are the mean  $\pm$  SE of  $n=4$  independent experiments performed with 5 replicates/treatment. All data are expressed as the percentage of survival of non-irradiated control cells (=100%). Statistically significant differences between the oligonucleotide treatments are indicated (\* $P < 0.05$ ).

**Figure 5-** MCF-7 cell transformation with PKC  $\delta$  dominant negatives. MCF-7 cells ( $1 \times 10^6$ /35 mm<sup>2</sup> dish) were transfected with 10  $\mu$ g pcDNA3-neo (EMPTY) or 13.7  $\mu$ g pcDNA3-neo-PKC $\delta$  dominant negative (PKC $\delta$  dn) (normalized for neomycin resistance gene copy number). Post-transfection (96 h) culture medium was exchanged with DMEM/10% FBS containing 400  $\mu$ g/ml G418. A, After 11 days of growth in selection medium cells were harvested and counted using a hemocytometer. B, After 11 days of growth in selection medium whole cell extracts were prepared. Proteins (30  $\mu$ g/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with a PKC  $\delta$  and Bcl-2 antibodies. Data are the mean  $\pm$  SE of  $n=3$  experiments (A). Blot is typical of  $n=3$  experiments (B).

**Figure 6-** PKC  $\delta$  oligonucleotide induced DNA damage. MDA-MB-231 cells ( $1.1 \times 10^5$ / 35 mm<sup>2</sup> dish) were treated with nothing (C), lipofectin (L), scrambled oligonucleotide (scr) or PKC  $\delta$  oligonucleotide (PKC $\delta$ ). Following treatment (24 h), cells maintained on ice were treated with 1.5 Gy IR and prepared for comet analysis. The number of comets with tail moments in ranges between 0-2 and 100-200 were plotted. Data are pooled from n=3 independent experiments with 80 comets scored per treatment per experiment. Statistically significant differences ( $P < 0.05$ ) were present between treatment groups receiving PKC  $\delta$  oligonucleotide versus nucleotide scrambled sequence PKC oligonucleotide and between all irradiated versus non-irradiated treatment groups. B, MDA-MB-231 cells were treated as described in A. Comet images representative of control (C), radiation (IR), scrambled oligonucleotide (scr) and PKC  $\delta$  oligonucleotide treatment groups are shown at 63X magnification with the corresponding tail moment (TM) indicated.





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